Analytical chip with an array of immobilized specific recognition elements for the determination of clinically relevant bacteria and analytical method based thereon

About 50 different bacteria are responsible for over 90 % of current occurrence of bacterial infections causing diseases of humans. Therefore, an analysis platform is highly desirable, that would be operable for analyzing a provided sample simultaneously for one or several bacterial targets in a uniform format, with a short time-to-result. all or for a significant part of these bacteria or compounds respectively markers derived therefrom.

Bacterial pathogens are usually identified by time-consuming biochemical testing, e.g. of metabolic products, cell constituents or cell wall constituents, monitoring of bacterial growth in selected culture media, etc. These techniques, requiring the cultivation of the samples, need several hours to days for growth and isolation of the organisms followed by, e.g., measurement of enzymatic activity, of anti-microbial susceptibilities, or of concentrations of metabolites. During this time, a tentative diagnosis is made on the basis of clinical presentation. Patients receive empirical treatments with broad-spectrum antibiotics as immediate medical action. This increases costs and promotes antibiotic resistance, decreasing the usefulness of entire antibiotic families.

Molecular techniques allow rapid and sensitive identification of slow growing (*M. tuberculosis*) or even non-cultivable bacteria (*M. genavense*; B. Hirschel , H.R. Chang, N. Mach, P.F. Piguet, J. Cox, J.D. Piguet, M.T. Silva, L. Larsson, P.R. Klatser and J.E. Thole. 1990. "Fatal infection with a novel, unidentified mycobacterium in a man with the acquired immunodeficiency syndrome." New England.Journal.of.Medicine 323:109-113; E.C. Bottger, A. Teske, P. Kirschner, S. Bost, H.R. Chang, V. Beer and B. Hirschel. 1992. "Disseminated "*Mycobacterium genavense*" infection in patients with AIDS". Lancet 340:76-80). However, they require prior pathogen isolation and/or careful sample preparation. With the notable exception of sequencing, these methods can be defined as "closed" and "serial" because each assay identifies only a single specific pathogen.

Nucleic acid probes, labeled with enzymes, antigenic substrates, chemiluminescent moieties, or radioisotopes, can bind with high specificity to complementary sequences of a target

nucleic acid. The sensitivity of the assay depends on the size of the probe, the degree of homology with its target and the labeling method. Present technology typically requires cultivation followed by enzymatic amplification by culture or by enzymatic approaches. The latter include thermal cycle strategies such as the polymerase chain reaction (PCR), as well as isothermal strategies such as transcription-mediated amplification (TMA, Accuprobe®, Gen Probe, San Diego, CA) or strand displacement amplification (SDA, Becton-Dickinson, New Jersey, NJ). These target amplification strategies suffer from the possibility of self-contamination, since the product of one reaction serves as template for the next. As organism identification is more reproducible from pure culture or isolated colonies than from direct specimens, results are usually not available the same day.

Instead of amplifying the template itself, alternate strategies amplify signal from the template, reducing the risk of contamination. One such signal amplification strategy uses a proprietary conformation-sensitive endonuclease (Invader™, Third Wave Technologies, Madison, WI). This strategy is successful in identifying microorganism species (D.J.Marshall, L.M. Heisler, V. Lyamichev, C. Murvine, D.M. Olive, G.D. Ehrlich, B.P. Neri and M. de Arruda. 1997. « Determination of hepatitis C virus genotypes in the United States by cleavase fragment length polymorphism analysis. » Journal.of.Clinical.Microbiology. *35*:3156-3162.), antibiotic resistance (S. Sreevatsan, J.B. Bookout, F.M. Ringpis, S.L. Mogazeh, B.N. Kreiswirth, R.R. Pottathil and R.R. Barathur. 1998. « Comparative Evaluation of Cleavase Fragment Length Polymorphism With PCR-SSCP and PCR-RFLP to Detect Antimicrobial Agent Resistance in Mycobacterium tuberculosis. » Mol.Diagn. *3*:81-91), and mutations (S. Rossetti, S. Englisch, E. Bresin, P.F. Pignatti and A.E. Turco. 1997. « Detection of mutations in human genes by a new rapid method: cleavage fragment length polymorphism analysis (CFLPA). » Molecular.& Cellular.Probes. *11*:155-160.), all by genotype identification. The strength of the method lies in isothermal detection and absence of amplicon contamination. However, this powerful signal amplification technology does not rival PCR amplification rates and lacks parallelism and automation. Other signal amplification strategies include ligase-chain reaction (LCR, Abbott, IL), branched DNA (bDNA, Chiron, Emeryville, CA), or label-antibody-label stacks.

Automated analysis of nucleic acid sequences remains a marginal strategy for bacterial identification. Although various detection methods have been automated, all suffer from low sensitivity and require some amplification before detection can take place. To date, most

molecular techniques rely on PCR. Automated PCR coupled with EIA detection has been used for the identification of several microorganism (COBAS AMPLICOR™, Roche): *M. tuberculosis* (S.X. Wang and L. Tay. 1999. « Evaluation of three nucleic acid amplification methods for direct detection of Mycobacterium tuberculosis complex in respiratory specimens. » J.Clin.Microbiol. *37*:1932-1934.), *C. trachomatis* (R. Pasternack, P. Vuorinen and A. Miettinen. 1999. « Comparison of a transcription-mediated amplification assay and polymerase chain reaction for detection of Chlamydia trachomatis in first-void urine. » Eur.J.Clin.Microbiol.Infect.Dis. *18*:142-144.), or viruses like HSV-2 (J. Groen, B. Hersmus, H.G. Niesters, W. Roest, G. van Dijk, W. van der Meijden and A.D. Osterhaus. 1999. « Evaluation of a fully automated glycoprotein G-2 based assay for the detection of HSV-2 specific IgG antibodies in serum and plasma. » J.Clin.Virol. *12*:193-200.), HIV, hepatitis B or C (W.K. Roth, M. Weber and E. Seifried. 1999. « Feasibility and efficacy of routine PCR screening of blood donations for hepatitis C virus, hepatitis B virus, and HIV-1 in a blood-bank setting. » Lancet *353*:359-363; A. Doglio, C. Laffont, F.X. Caroli-Bosc, P. Rochet and J. Lefebvre. 1999. « Second generation of the automated Cobas Amplicor HCV assay improves sensitivity of hepatitis C virus RNA detection and yields results that are more clinically relevant. » J.Clin.Microbiol. *37*:1567-1569.). Once again, these technologies are targeted to and optimized for the detection of only a single suspected microorganism per assay and yield only qualitative results.

Real-time PCR offers interesting improvements to clinical microbiology, including shorter turn-around time and reduced risk of amplicon contamination. On-line fluorescence monitoring of PCR-generated amplicons is achieved, e.g., through molecular beacons (S. Tyagi and F.R. Kramer. 1996. « Molecular beacons: probes that fluoresce upon hybridization. » Nature Biotechnology *14*:303-308.), fluorescence-resonance energy transfer between probes hybridized to adjacent sites on the amplicon (C.A. Gelfand, G.E. Plum, S. Mielewczyk, D.P. Remeta and K.J. Breslauer. 1999. « A quantitative method for evaluating the stabilities of nucleic acids. » Proc.Natl.Acad.Sci.U.S.A. *96*:6113-6118.; N. Ota, K. Hirano, M. Warashina, A. Andrus, B. Mullah, K. Hatanaka and K. Taira. 1997. « Structural analysis of nucleic acids by using fluorescence resonance energy transfer (FRET). » Nucleic.Acids.Symposium.Series. 207-208.), or TaqMan™ probes (C.A. Heid, J. Stevens, K.J. Livak and P.M. Williams. 1996. « Real time quantitative PCR. » Genome Research. *6*:986-994.). Rugged systems for very rapid field-testing (P. Belgrader, W. Benett, D. Hadley, J. Richards, P. Stratton, R.Jr. Mariella and F. Milanovich. 1999. « PCR detection of bacteria

in seven minutes. » Science *284*:449-450.) integrate sample preparation, amplification and detection in the same cartridge (GeneXpert™ System, Cepheid, Sunnyvale, CA). This is close to a practical point-of-care instrument. Its major drawback is the need to define the pathogen(s) to be tested and select the appropriate cartridge(s). Thus, an "open diagnosis" would require one specific cartridge for each putative pathogen.

To date, a single commercially available system allows parallel determination of up to 96 different reactions for the same patient using a 96-wells plate format (ABI PRISM 5700/7700, Perkin-Elmer BioSystems, Foster City, CA) or a 384-wells plate format (ABI Prism 7900 HT). However, its costs will not permit routine real-time diagnosis testing. Multiplex PCR, using numerous primer pairs and probes within a single reaction tube, can detect nucleic acid fragments from different organisms. Unfortunately, its development is tedious and the modest overall decrease in the total number of wells will not lead to significant cost savings.

16S ribosomal RNA (16S-rRNA) gene sequencing has been used successfully for phylogenetic analysis (M.C. Enright, P.E. Carter, I.A. MacLean and H. McKenzie. 1994. « Phylogenetic relationships between some members of the genera Neisseria, Acinetobacter, Moraxella, and Kingella based on partial 16S ribosomal DNA sequence analysis. » International.Journal.of.Systematic.Bacteriology. 44:387-391.) as well as for bacterial identification (MicroSeq® kit, Perkin-Elmer Biosystems, Foster City, CA). Using universal primers to amplify discriminant sequences, this method can detect unexpected or even previously unknown pathogens (T. whippeli (D.A. Relman, T.M. Schmidt, R.P. MacDermott and S. Falkow. 1992. « Identification of the uncultured bacillus of Whipple's disease. » New England.Journal.of.Medicine 327:293-301.)). However, also because of universal primers, 16S-rRNA sequencing requires a pure culture of the pathogen before DNA extraction or amplification of the genetic material contained in a body fluid sample (such as blood, cerebral spinal fluid, etc.), after sterilization of the sample. PCR and sequencing procedures require an additional 24h for identification.

Massively parallel hybridization based on a very large number of DNA probes as recognition elements immobilized in discrete measurement areas forming arrays of measurement areas provided on "chips" (usually simple glass or microscope slides), for specific analyte detection, is at present widely advocated as the diagnostic tool of the future.

For example in US-patent No. 5,445,934 (Affymax Technologies) arrays of oligonucleotides with a density of more than 1000 features on a square centimeter are described and claimed. The excitation and read-out of such arrays is based on classical optical arrangements and methods. The whole array can be illuminated simultaneously, using an expanded excitation light bundle, which, however, results in a relatively low sensitivity, the portion of scattered light being relatively large and scattered light or background fluorescence light from the glass substrate being also generated in those regions, where no oligonucleotides for binding of the analyte are immobilized. In order to limit excitation and detection to the regions of immobilized features and to suppress light generation in the adjacent regions, there is widespread use of confocal measurement arrangements, and the different features are analyzed sequentially by scanning. The consequences, however, are an increased amount of time for the read-out of a large array and a relatively complex optical set-up.

In the international patent application WO 97/29212 a method for identifying organisms, in especial Mycobacterium tuberculosis, based on the hybridization pattern of a sample and its comparison with a reference hybridization pattern, is claimed. Thereby, the nomenclature "hybridization pattern" is understood as the pattern of the locally resolved signals from an array of measurement areas recorded by a detector, as a consequence of the interaction between immobilized oligonucleotides with the sample. Besides others, also optical detection methods using fluorescence labels attached to the target polynucleotides to be detected are described. These optical methods disclosed in WO 97/29212, however, are based on classical optical configurations which cannot provide sufficient sensitivity for analyzing small amount of sample material or for detecting analytes at very low concentrations. As a consequence, in that disclosure it is strongly preferred that a nucleic acid sample is amplified prior to hybridization. Accordingly, no hint is given in that patent application to a simultaneous determination of target polynucleotides from different organisms or bacteria on one chip, but only to a determination of one or more species-specific polymorphisms.

In summary, a number of promising novel strategies are currently being under development to identify pathogens, such as bacteria, based on determination of their nucleic acid sequences. Most of them require bacterial culturing, followed by enzymatic amplification steps, with the prerequisite of knowledge of the target organism respectively sequence. In the context of this invention such a method, wherein the properties of the target analyte have to be exactly known, shall be called a "closed" analysis method, in contrast, in contrast to "open" analysis

methods as provided by the present invention not requiring exact knowledge of the complete target sequence (see below). These amplification steps limit the speed of analysis and the possibilities for multiple parallel identification. There is a need for new techniques and methods which do not require such amplification steps, and which allow for the parallel, simultaneous identification of (one or multiple) organisms (bacteria), e.g. by identification of their 16S-rRNA contained in a sample, by a single measurement and its comparison with reference data.

A significant improvement of detection limits can be achieved, when, instead of the classical detection configurations (for example based on epi-fluorescence excitation as used for most scanners), the determination of an analyte is based on its interaction with the evanescent field, which is, for example, associated with light guiding in an optical waveguide, wherein biochemical or biological recognition elements for the specific recognition and binding of the analyte molecules are immobilized on the surface of the waveguide. When a light wave is coupled into an optical waveguide surrounded by optically rarer media, i.e. media of lower refractive index, the light wave is guided by total reflection at the interfaces of the waveguiding layer. In that arrangement, a fraction of the electromagnetic energy penetrates the media of lower refractive index. This portion is termed the evanescent field. The strength of the evanescent field depends to a very great extent on the thickness of the waveguiding layer itself and on the ratio of the refractive indices of the waveguiding layer and of the media surrounding it. In the case of thin waveguides, i.e. layer thicknesses that are the same as or smaller than the wavelength of the light to be guided, discrete modes of the guided light can be distinguished. As an advantage of such methods, the interaction with the analyte is limited to the penetration depth of the evanescent field into the adjacent medium, being of the order of some hundred nanometers, and interfering signals from the depth of the (bulk) medium can be mainly avoided. The first proposed measurement arrangements of this type were based on highly multi-modal, self-supporting single-layer waveguides, such as fibers or plates of transparent plastics or glass, with thicknesses from some hundred micrometers up to several millimeters.

For a further improvement of the sensitivity and simultaneously for an easier manufacturing in mass production, planar thin-film waveguides have been proposed. In the simplest case, a planar thin-film waveguide consists of a three-layer system: support material (substrate),

waveguiding layer, superstrate (respectively the sample to be analyzed), wherein the waveguiding layer has the highest refractive index.

Different methods of analyte determination in the evanescent field of lightwaves guided in optical film waveguides can be distinguished. Based on the applied measurement principle, for example, it can be distinguished between fluorescence, or more general luminescence methods, on one side and refractive methods on the other side. In this context methods for generation of surface plasmon renonance in a thin metal layer on a dielectric layer of lower refractive index can be included in the group of refractive methods, if the resonance angle of the launched excitation light for generation of the surface plasmon resonance is taken as the quantity to be measured. Surface plasmon resonance can also be used for the amplification of a luminescence or the improvement of the signal-to-background ratios in a luminescence measurement. The conditions for generation of a surface plasmon resonance and the combination with luminescence measurements, as well as with waveguiding structures, are described in the literature, for example in US-patents No. 5,478,755, No. 5,841,143, No. 5,006,716, and No. 4,649,280.

In this application, the term "luminescence" means the spontaneous emission of photons in the range from ultraviolet to infrared, after optical or other than optical excitation, such as electrical or chemical or biochemical or thermal excitation. For example, chemiluminescence, bioluminescence, electroluminescence, and especially fluorescence and phosphorescence are included under the term "luminescence".

In case of the refractive measurement methods, the change of the effective refractive index resulting from molecular adsorption to or desorption from the waveguide is used for analyte detection. This change of the effective refractive index is determined, in case of grating coupler sensors, from changes of the coupling angle for the in- or out-coupling of light into or out of the grating coupler sensor, in case of interferometric sensors from changes of the phase difference between measurement light guided in a sensing branch and a referencing branch of the interferometer.

The aforesaid refractive methods have the advantage, that they can be applied without using additional marker molecules, so-called molecular labels. The disadvantage of these label-free methods, however, is, that the achievable detection limits are limited to pico- up to nanomolar

concentration ranges, dependent on the molecular weight of the analyte, due to lower selectivity of the measurement principle, which is not sufficient for many applications of modern trace analysis, for example for diagnostic applications.

For achieving lower detection limits, luminescence-based methods appear as more adequate, because of higher selectivity of signal generation. In this arrangement, luminescence excitation is limited to the penetration depth of the evanescent field into the medium of lower refractive index, i.e to the immediate proximity of the waveguiding area, with a penetration depth of the order of some hundred nanometers into the medium. This principle is called evanescent luminescence excitation.

By means of highly refractive thin-film waveguides, based on an only some hundred nanometers thin waveguiding film on a transparent support material, the sensitivity could be increased considerably during the last years. In WO 95/33197, for example, a method is described, wherein the excitation light is coupled into the waveguiding film by a relief grating as a diffractive optical element. The isotropically emitted luminescence from substances capable of luminescence, that are located within the penetration depth of the evanescent field, is measured by adequate measurement devices, such as photodiodes, photomultipliers or CCD cameras. The portion of evanescently excited radiation, that has back-coupled into the waveguide, can also be out-coupled by a diffractive optical element, like a grating, and be measured. This method is described, for example, in WO 95/33198.

Configurations based on thin-film waveguides for the (simultaneous) determination of multiple analytes in a supplied sample are described (e.g. in WO 96/35940), the proposed techniques and analysis methods are always related to a unique assignment and application of one biological or biochemical or synthetic recognition element for one particular analyte to be determined. However, no concept is described for the analysis and identification of one analyte (such as 16S-rRNA) by a plurality of recognition elements, upon generation of a signal pattern leading to the identification of the analyte.

The present invention solves the need defined above. It provides an analytical chip and an analytical method based thereon enabling to analyze a provided sample simultaneously for 16S-rRNA from a multitude of different organisms (bacteria). In contrast to all cited prior art,

the invention provides the capability of determining not only one, but a multitude (i.e. two or more) organisms, especially bacteria, simultaneously using one analytical chip according to the invention in an inventive analysis method, without the need for a target amplification. The analytical method according to the invention is readily available for automation, using a commercial analytical system (ZeptoREADER™, Zeptosens AG, Witterswil, Switzerland).

The invention is particularly useful for a fast and easy identification of bacteria by genotypic characterization in a provided sample. Due to the advantageous properties of an evanescent field measurement platform as the sensing platform of an analytical chip according to the invention, considerably less steps of sample preparation are required. Identification of a bacterium even in a complex biological sample is enabled. As a consequence of the lower number of required work-up steps, which are each associated with the risk of the introduction of experimental error, bias and variation, the reliability and confidence into the results, as well as though-put of an analytical method using the analytical chip according to the invention, is considerably increased in comparison to the known methods. As a further consequence, the analytical chip according to the invention allows for a simultaneous quantitative determination of one or more different bacterial 16S-rRNA in a liquid sample, i.e. with an experimental variation of less than 50 %, preferably of less than 20 %, most preferably of less than 10 %. Thereby, the achievable low degree of experimental variation is of course dependent on the amount of available 16S-rRNA to be detected (i.e. a lower variation can be achieved if more of the 16S-rRNA to be detected is available). In contrast to the known analytical equipment and analysis methods based thereon, especially due to the lower number of required work-up steps (especially of biological / and or biochemical work-up steps typically inducing a large variability of measurement results), and the avoidance of enzymatic and signal amplification steps, surprisingly even a quantitative determination of the amount respectively concentration of the one or more different bacteria in the original sample from where the liquid sample containing said one or more different 16S-rRNA have been derived, is enabled.

The invention includes the use of genomic target analytes other than 16S-rRNA for identification of organisms (e.g. bacteria), such as 23S-rDNA, Internal Transmission Sequences (ITS) and the like, as they are known to a person skilled in the art.

The selection of characteristic subsequences of a 16S-rRNA to be detected, and consequently of their complementary recognition elements (= capture probes to be immobilized), may be based, e.g., on sequence information, GC-content, positioning in a stable region (i.e., a region without frequent mutational variations), physico-chemical parameters, such as the melting temperature, and structural information (e.g. about loops, bulges, etc.). The selection can be supported and optimized using statistical and other mathematical methods, such as hierarchical cluster analysis (HCA), principal component analysis (PCA), and artificial neural networks (ANN). These methods are described in the literature.

In addition, the named statistical or mathematical analysis methods, like PCA, HCA, and ANN, are used for optimization and especially reduction or minimization of the set (number) of different capture probes for a certain 16S-rRNA to be detected for identification of the related organism (bacterium).

The interpretation of the results, i.e., the assignment of an observed hybridization or binding pattern, may be based not only on a simple comparison with reference or library data, but supported by the same type of statistical and mathematical methods (e.g. hierarchical cluster analysis, principal component analysis, artificial neural networks, etc.). The results can be utilized for generating data libraries, as well as comparison with data libraries can be performed for identification of an organism (bacterium) based on the measured hybridization or binding patterns. Typically, the mentioned statistical respectively mathematical methods provide a ranking of probabilities of the identity of an organism to be identified with reference organisms, based on the comparison of the actual binding (respectively hybridization) patterns with reference patterns.

A first subject of the invention is an analytical chip for the simultaneous determination of one or more different bacterial 16S-rRNA in a liquid sample

comprising

- an evanescent field measurement platform, e.g. an optical waveguide, as a solid carrier and

- a plurality of specific recognition elements immobilized in discrete measurement areas of known location forming an array of measurement areas on said evanescent field measurement platform,

wherein

- a multitude (i.e. 2 or more) of different specific recognition elements is immobilized in discrete measurement areas for the recognition and detection of each different 16S-rRNA, different recognition elements being specific for different subsequences of the 16S-rRNA to be detected, which are not directly adjacent and not overlapping in the sequence of said 16S-rRNA, and
- and said analytical chip is operable for the detection of 16S-rRNA in the evanescent field of the evanescent field measurement platform, without an amplification (e.g. by polymerase chain reaction PCR or linear amplification "T7") of the polynucleotide sequences contained in the sample.

Under "linear amplification "T7"" it shall be understood a linear amplification using an in vitro transcription initiated by a T7 promoter.

In the spirit of this invention, spatially separated or discrete measurement areas shall be defined by the closed area that is occupied by binding partners, such as polynucleotides, immobilized thereon, for determination of one or multiple analytes in one or multiple samples in a bioaffinity assay, such as a hybridization assay. These areas can have any geometry, for example the form of circles, rectangles, triangles, ellipses etc.

It is preferred that the one or more bacterial 16S-rRNA to be detected are derived from bacteria selected from the group comprising, e.g.: *Achromobacter xylosoxidans, Acinetobacter baumannii, Acinetobacter calcoaceticus, Acinetobacter junii, Acinetobacter wolfii, Actinobacillus sp, Actinomyces israelii, Actinomyces meyeri, Actinomyces odontolyticus, Actinomyces sp, Aerococcus viridans, Aeromonas caviae, Aeromonas hydrophilia, Aeromonas sobria, Agrobacterium radiobacter, Alcaligenes denitrificans, Alcaligenes faecalis, Alcaligenes sp, Alcaligenes xylosoxydans, Bacillus sp, Bacteroides bivius, Bacteroides buccae, Bacteroides caccae, Bacteroides denticola, Bacteroides disiens, Bacteroides distasonis, Bacteroides fragilis, Bacteroides oralis, Bacteroides oris, Bacteroides ovatus, Bacteroides stercoris, Bacteroides thetaiotomicron, Bacteroides uniformis,*

Bacteroides ureolyticus, Bacteroides vulgatus, Bifidobacterium sp, Bordetella bronchiseptica, Brucella melitensis, Burkholderia cepacia, Burkholderia picketti, Burkholderia pseudomallei, Campylobacter coli, Campylobacter fetus, Campylobacter jejuni, Campylobacter sp, Capnocytophaga canimorsus, Capnocytophaga ochracea, Capnocytophaga sp, Chryseomonas luteola, Citrobacter amalonaticus, Citrobacter braakii, Citrobacter diversus, Citrobacter freundii, Citrobacter koseri, Citrobacter sp, Clostridium bifermentans, Clostridium butyricum, Clostridium clostridiiforme, Clostridium paraputrificum, Clostridium perfringens, Clostridium ramosum, Clostridium septicum, Clostridium tertium, Clostridiumi innocuum, Comamonas acidovora, Corynebacterium aquaticum, Corynebacterium bovis, Corynebacterium jeikeium, Corynebacterium minutissimum, Corynebacterium sp, Eikenella corrodens, Empedobacter brevis, Entereococcus casseliflavus, Enterobacter aerogenes, Enterobacter agglomerans, Enterobacter amnigenus, Enterobacter cloacae, Enterococcus avium, Enterococcus durans, Enterococcus faecalis, Enterococcus faecium, Enterococcus gallinarium, Enterococcus raffinosus, Escherichia coli, Eubacterium aerofaciens, Eubacterium lentum, Eubacterium limosum, Flavobacterium breve, Flavobacterium meningosepticum, Flavobacterium sp, Fusobacterium sp, Fusobacterium mortiferum, Fusobacterium necrophorum, Fusobacterium nucleatum, Fusobacterium varium, Gardnerella vaginalis, Gemella haemolysans, Gemella morbillorum, Gemella sp, Haemophilus aphrophilus, Haemophilus influenzae, Haemophilus parainfluenzae, Haemophilus paraphrophilus, Hafnia alvei, Kingella sp, Klebsiella ornithinolytica, Klebsiella oxytoca, Klebsiella ozaenae, Klebsiella pneumoniae, Kluyvera sp, Lactobacillus acidophilus, Lactobacillus catenaforme, Lactococcus cremoris, Lactococcus lactis, Legionella pneumophila, Leptotrichia buccalis, Leuconostoc sp, Listeria monocytogenes, Moraxella catarrhalis, Moraxella osloensis, Moraxella phenylpyruvica, Moraxella sp, Morganella morganii, Mycobacterium avium, Mycobacterium genavense, Mycobacterium tuberculosis, Mycobacterium avium-intracellulare, Mycoplasma sp, Myroides odoratum, Neisseria cinerea, Neisseria flavescens, Neisseria meningitidis, Neisseria mucosa, Neisseria sp, Neisseria subflava, Nocardia asteroides, Nocardia sp, Ochrobactrum anthropi, Pasteurella multocida, Peptostreptococcus anaerobius, Peptostreptococcus asaccharolyticus, Peptostreptococcus magnus, Peptostreptococcus micros, Peptostreptococcus prevotii, Prevotella bivia, Prevotella buccae, Prevotella loescheii, Propionibacterium acnes, Propionibacterium granulosum, Proteus mirabilis, Proteus penneri, Proteus vulgaris, Providencia rettgeri, Providencia sp, Providencia stuartii, Pseudomonas aeroginosa, Pseudomonas alcaligenes, Pseudomonas diminuta, Pseudomonas fluorescens, Pseudomonas paucimobilis, Pseudomonas putida,

Pseudomonas sp, Pseudomonas stutzeri, Pseudomonas vesicularis, Salmonella enteritidis, Salmonella paratyphi, Salmonella typhi, Salmonella typhimurium, Serratia fonticola, Serratia marcescens, Serratia odorifera, Serratia sp, Shigella dysenteria, Shigella flexneri, Shigella sonnei, Sphingomonas paucimobilis, Staphylococcus aureus, Staphylococcus auricularis, Staphylococcus capitis, Staphylococcus caprae, Staphylococcus chromogenes, Staphylococcus cohnii, Staphylococcus epidermidis, Staphylococcus haemolyticus, Staphylococcus hominis, Staphylococcus intermedius, Staphylococcus kloosii, Staphylococcus lugdunensis, Staphylococcus saccharolyticus, Staphylococcus saprophyticus, Staphylococcus sciuri, Staphylococcus simulans, Staphylococcus warneri, Staphylococcus xylosus, Stenotrophomonas maltophilia, Stomatococcus mucilaginosus, Streptococcus acidiminimus, Streptococcus adjacens, Streptococcus agalactiae, Streptococcus anginosus, Streptococcus bovis, Streptococcus canis, Streptococcus constellatus, Streptococcus cremoris, Streptococcus crista, Streptococcus defectivus, Streptococcus dysgalactiae, Streptococcus equinus, Streptococcus equisimilis, Streptococcus intermedius, Streptococcus lactis, Streptococcus mitis, Streptococcus mutans, Streptococcus oralis, Streptococcus pneumoniae, Streptococcus pyogenes, Streptococcus salivarius, Streptococcus sanguis, Streptococcus alpha-hemolyticus, Streptococcus beta-hemolyticus, Veillonella parvula, Veillonella sp, Yersinia enterocolitica, and the like.

Although polynucleotides are the most obvious recognition elements for recognition of and hybridizing with complementary subsequences of a 16S-rRNA to be detected, other possible recognition elements for RNA have been described, which are, for example, based on RNA-ligand interactions, especially on RNA-protein interactions. This is attributed to the observation that unlike DNA, which mostly occurs as a base-paired duplex of complementary strands, RNA is almost always folded from a single strand. Electrostatic repulsion between sections of the highly charged ribose-phosphate backbone are regarded as a driving force for RNA folding. As a consequence, the RNA can assume secondary structures which can be recognized, for example, by proteins (R.A. Zimmermann, et al. 2000. "How ribosomal proteins and rRNA recognize one another". The ribosome: Structure, function, antibiotics, and cellular interactions. (R.A. Garrett et al. eds.), pp. 93-104, ASM Press, Washington, D.C.) and also by specifically targeted synthetic molecules, like antiviral drugs (D.J. Ecker, R.H. Griffey, 1999. "RNA as a small molecule drug target: doubling the value of genomics." Drug Discovery Today 4, 420 – 429) that bind to viral-specific RNA.

Accordingly, for one group of embodiments of an analytical chip according to the invention, it is characteristic that the immobilized specific recognition elements are selected from the group comprising, e.g., natural and synthetically fabricated polynucleotides, polynucleotides with artificial bases and / or artificial carbohydrates, peptides, peptide nucleic acids ("PNA"s), PNA's with artificial bases, Locked nucleic acids ® (LNAs) (Exiqon, DK-2950 Vedbaek, Denmark), proteins (e.g. antibodies), ribozymes, and aptamers.

According to the recognition elements, another group of embodiments of the analytical chip can be distinguished, wherein the immobilized specific recognition elements are selected from the group of antibiotics-based, DNA- or RNA-selective recognition elements comprising, e.g., macrolide antibiotics (e.g. erythromycin, azithromycin, streptogramin), aminoglycoside antibiotics (e.g. neomycin, paromomycin, lividomycin, gentamycin), and peptide antibiotics (e.g. thiostreptone, micrococcin) and the like.

Preferred, however, is an embodiment of an analytical chip for the simultaneous determination of one or more different bacterial 16S-rRNA in a liquid sample

comprising

- an evanescent field measurement platform, e.g. an optical waveguide, as a solid carrier and
- a plurality of polynucleotides immobilized in discrete measurement areas of known location forming an array of measurement areas on said evanescent field measurement platform,

wherein

- a multitude (i.e. 2 or more) of different polynucleotides is immobilized in discrete measurement areas for the detection of each different 16S-rRNA, the sequences of the immobilized polynucleotides being essentially complementary to different subsequences of the 16S-rRNA to be detected, which are not directly adjacent and not overlapping in the sequence of said 16S-rRNA, and
- and said analytical chip is operable for the detection of 16S-rRNA in the evanescent field of the evanescent field measurement platform, without an amplification (e.g. by polymerase chain reaction PCR or linear amplification "T7") of the polynucleotide sequences contained in the sample.

„Essential complementarity" between the sequences of the immobilized polynucleotides and subsequences of the 16S-rRNA shall mean, that these sequences are complementary except for no more than 10 % mismatches. Accordingly, also bacterial 16S-rRNA with corresponding point mutations in the subsequences to be hybridized can be analyzed with an analytical chip according to the invention and with a corresponding analytical method according to the invention (see below), using said analytical chip. Dependent on the choice of the sequence of the immobilized polynucleotides (capture probes), with respect to the 16S-rRNA to be detected, such mutations may be detected using an analytical chip according to the invention, or they may be disregarded.

In the context of this patent application, "hybridization" between essentially complementary poynucleotides is understood as a special form of binding between a specific recognition element and the analyte (or another specific binding partner), identical to or comparable with the Watson / Crick base pair interaction.

Preferably, the immobilized polynucleotides for the detection of the bacterial 16S-rRNA have a length of 5 – 500, more preferably of 10 – 100, most preferably of 10 - 30 bases.

These immobilized polynucleotides (capture probes) may have similar length (number of base pairs), or different capture probes may also differ in length.

In typical embodiments of the analytical chip according to the invention, the plurality of immobilized polynucleotides comprises 2 – 20 different polynucleotides which are essentially complementary to different subsequences of the same bacterial 16S-rRNA to be detected.

Due to the high sensitivity provided by analytical chip according to the invention, not requiring an amplification (e.g. by polymerase chain reaction PCR or linear amplification "T7") of the polynucleotide sequences contained in the sample, the risk of falsifications occurring during the amplification process can be avoided. Therefore, in general a lower number of different immobilized specific recognition elements (respectively of polynucleotides for the preferred embodiments) is required for the unambiguous detection of a certain 16S-rRNA than for known analytical chips, which in general can only be applied in combination with the amplification of the biological material contained in a sample. It is

preferred, that the plurality of immobilized polynucleotides (or more generally: of specific recognition elements) comprises less than 10, preferably less than 5 different polynucleotides (respectively specific recognition elements) which are essentially complementary to different subsequences (respectively: can specifically bind to different subsequences) of the same bacterial 16S-rRNA to be detected.

Besides the obvious advantage of reduced costs for the production of an analytical chip with only a small number of specific recognition elements for the target (16S-rRNA) to be determined, further advantages are a sharper level of discrimination whether hybridization (respectively in case of recognition elements other than polynucleotides: whether specific binding) occurred or not, and a less ambiguous result of the comparison of an obtained sample hybridization pattern with reference data. Due to the small required number of capture probes (specific recognition elements in general, polynucleotides in especial), a large number of different 16S-rRNA can be addressed on one and the same analytical chip.

It is well-known that a bacterial 16S-rRNA contains both subsequences that are characteristic for a certain genus and common for all species of that genus, and other subsequences characteristic for a certain or even for a strain. Accordingly, as one possible embodiment of an analytical chip according to the invention the sequences of the multitude of immobilized polynucleotides for detection of a 16S-rRNA are essentially complementary to subsequences indicative for the genus of the bacterium from which said 16S-rRNA to be detected has been derived. Characteristic for other possible embodiments is, that the sequences of the multitude of immobilized polynucleotides for detection of a 16S-rRNA is essentially complementary to subsequences indicative for the species and / or strain of the bacterium from which said 16S-rRNA to be detected has been derived. Still another embodiment is characterized in that the multitude of immobilized polynucleotides for detection of a 16S-rRNA comprises both polynucleotides with a sequence essentially complementary to subsequences indicative for the genus type and polynucleotides with a sequence essentially complementary to the species and / or strain of the bacterium from which said 16S-rRNA to be detected has been derived. The possibility to identify a genus based on selected "signature" sequences allows the identification or exclusion of unknown species that are not part of an established pattern library.

Besides high sensitivity, the evanescent field measurement platform provides to the analytical chip according to the invention as a further advantage that signal generation and detection is confined to the sensing surface and that the risk of interfering signals from the sample matrix in the bulk medium, outside the penetration depth of the evanescent field, is eliminated. As a consequence, a sample to be analyzed can be embedded within almost any medium, and considerably less sample preparation (for example for simplification of the sample matrix) is required compared to analysis on conventional supports like microscope slides, using, e.g., confocal epifluorescence detection. Without restriction of generality, the liquid sample may comprise a complex biological matrix of the group of human and animal cell extracts, extracts of human and animal tissue, such as organ, skin or bone tissue, and of body fluids or their components, such as blood, serum, plasm, lymph, synovia, tear liquid, sweat, milk, sperm, sputum, cerebral spinal fluid, gastric juice, intestinal contents, urine, and stool. For example, the sample may be a clinical sample (e.g. from a patient's blood or body secreta) and may be screened for a variety of bacteria that could be contained therein.

The evanescent field measurement platform may be operable to work with a single total reflection for a launched light ray, like a prism. Then an expanded bundle of essentially parallel light rays would be launched in such a way that it would hit the surface on which an array of measurement areas would be accommodated under an angle matching the condition for total reflection.

"Essentially parallel" shall mean, that the angle of divergence or convergence of a light bundle is not more than 1°.

However, it is much more convenient, if the evanescent field measurement platform can provide multiple points of total reflection, with multiple isolated locations of generation of an evanescent field in the outside medium, or even a continuous evanescent field zone, as it is characteristic for low-mode waveguides.

Therefore, it is strongly preferred if the evanescent field measurement platform comprises an optical waveguide. The optical waveguide may be continuous or be partitioned into discrete waveguiding areas.

It is preferred that the optical waveguide is provided as optical film waveguide with a first optically transparent layer (a) on a second optically transparent layer (b) with lower refractive index than layer (a). "Optical transparency" of a material shall mean that it is transparent at least at the wavelength of an irradiated excitation light, that can have a wavelength between the UV and near-IR spectral region (between 200 nm and 1200 nm). If, as a result of irradiation with excitation light, a luminescence or fluorescence is generated, the material should also be transparent at the wavelength of said luminescence or fluorescence, which also may be between 200 nm and 1200 nm.

Different embodiments of optical film waveguides, that are suitable for an analytical chip according to the invention as an evanescent field measurement platform, have been described in several international patent applications, such as WO 95/33197, WO 95/33198, WO 96/35940, WO 00/75644, WO 01/43875 (as part of flow cell arrangements), WO 01/79821, and WO 01/88511, that are included in this application in their full entirety. An example of such a thin-film is illustrated in Figure 4 of WO 01/43875.

It is preferred that the material of the second optically transparent layer (b) comprises, for example, silicates, such as glass or quartz, or a transparent thermoplastic or moldable plastic, preferably of the group comprising polycarbonate, polyimide, or polymethylmethacrylate, or polystyrene.

For generating an evanescent field as strong as possible at the surface of the optically transparent layer (a), it is desirable that the refractive index of the waveguiding, optically transparent layer (a) is significantly higher than the refractive index of the adjacent layers. It is especially advantageous, if the refractive index of the first optically transparent layer (a) is higher than 1.8.

The first optically transparent layer (a) can comprise, for example, TiO_2, ZnO, Nb_2O_5, Ta_2O_5, HfO_2, or ZrO_2. It is especially preferred, if the first optically transparent layer (a) comprises TiO_2, Ta_2O_5 or Nb_2O_5.

Besides the refractive index of the waveguiding optically transparent layer (a), its thickness is the second important parameter for the generation of an evanescent field as strong as possible at the interfaces to adjacent layers with lower refractive index. With decreasing thickness of

the waveguiding layer (a), the strength of the evanescent field increases, as long as the layer thickness is sufficient for guiding at least one mode of the excitation wavelength. Thereby, the minimum "cut-off" layer thickness for guiding a mode is dependent on the wavelength of this mode. The "cut-off" layer thickness is larger for light of longer wavelength than for light of shorter wavelength. Approaching the "cut-off" layer thickness, however, also unwanted propagation losses increase strongly, thus setting additionally a lower limit for the choice of the preferred layer thickness.

Preferred are layer thicknesses of the optically transparent layer (a) allowing for guiding only one to three modes at a given excitation wavelength. Especially preferred are layer thicknesses resulting in monomodal waveguides for this given excitation wavelength. It is understood that the character of discrete modes of the guided light does only refer to the transversal modes.

Resulting from these requirements, the thickness of the first optically transparent layer (a) is preferably between 40 and 300 nm. It is especially advantageous, if the thickness of the first optically transparent layer (a) is between 100 and 200 nm.

The amount of the propagation losses of a mode guided in an optically waveguiding layer (a) is determined to a large extent by the surface roughness of a supporting layer below and by the absorption of chromophores which might be contained in this supporting layer, which is, additionally, associated with the risk of excitation of unwanted luminescence in this supporting layer, upon penetration of the evanescent field of the mode guided in layer (a) into this supporting layer. Furthermore, thermal stress can occur due to different thermal expansion coefficients of the optically transparent layers (a) and (b). In case of a chemically sensitive optically transparent layer (b), consisting for example of a transparent thermoplastic plastics, it is desirable to prevent a penetration, for example through micro pores in the optically transparent layer (a), of solvents that might attack layer (b).

Therefore, it is advantageous, if an additional optically transparent layer (b') with lower refractive index than and in contact with layer (a), and with a thickness of 5 nm – 10 000 nm, preferably of 10 nm – 1000 nm, is located between the optically transparent layers (a) and (b). The purpose of the intermediate layer is a reduction of the surface roughness below layer (a) or a reduction of the penetration of the evanescent field, of light guided in layer (a), into the

one or more layers located below or an improvement of the adhesion of layer (a) to the one or more layers located below or a reduction of thermally induced stress within the optical sensor platform or a chemical isolation of the optically transparent layer (a) from layers located below, by sealing of micro-pores in layer (a) against the layers located below.

The in-coupling of excitation light into the optically transparent layer (a), to the measurement areas, can be performed using one or more optical in-coupling elements from the group comprising prism couplers, evanescent couplers comprising joined optical waveguides with overlapping evanescent fields, front face (distal end) couplers with focusing lenses arranged in front of a front face (distal end) of the waveguiding layer, and grating couplers. Thereby it is preferred that the in-coupling is performed using one or more grating structures (c) (acting as grating couplers), that are formed in the optically transparent layer (a).

Furthermore it is preferred, that out-coupling of light guided in the optically transparent layer (a) is performed using grating structures (c') that are formed in the optically transparent layer (a). Out-coupling of light guided in layer (a), at defined locations on the analytical chip, can, for example be beneficial to avoid reflections at the distal or lateral ends of the chip, which could lead to interference with the signals generated in the region of the measurement areas.

Grating structures (c) and (c') formed in the optically transparent layer (a) can have the same or different periodicity and may be arranged in parallel or not in parallel to one another.

Grating structures (c) and (c') can interchangeably be used as in-coupling and / or out-coupling gratings.

For given refractive indices of the waveguiding, optically transparent layer (a) and of the adjacent layers, the resonance angle for in-coupling of the excitation light, according to a well-defined resonance condition, is dependent on the diffraction order to be in-coupled, on the excitation wavelength and on the grating period. In-coupling of the first diffraction order is advantageous for increasing the in-coupling efficiency. Besides the number of the diffraction order, the grating depth is important for the amount of the in-coupling efficiency. As a matter of principle, the coupling efficiency increases with increasing grating depth. The process of out-coupling being completely reciprocal to the in-coupling, however, the out-coupling efficiency increases simultaneously, resulting in an optimum for the excitation of

luminescence in a measurement area located on or adjacent to the grating structure (c), the optimum being dependent on the geometry of the measurement areas and of the launched excitation light bundle. Based on these boundary conditions, it is advantageous, if the grating (c) has a period of 200 nm – 1000 nm and a modulation depth of 3 nm – 100 nm, preferably of 10 nm – 30 nm.

Furthermore, it is preferred that the ratio of the modulation depth of the grating to the thickness of the first optically transparent layer (a) is equal or smaller than 0.2.

It is also preferred that the grating structure (c) is a relief grating with a rectangular, triangular or semi-circular profile or a phase or volume grating with a periodic modulation in the essentially planar, optically transparent layer (a).

For most applications it is preferred, that the grating structure (c) is a diffractive grating with a uniform period. For some special applications, however, for example for in-coupling of light of different excitation wavelengths from different light sources, it can be advantageous if the grating structure (c) is a multi-diffractive grating.

For the geometrical arrangement of the arrays of measurement areas on an evanescent field measurement platform provided with in-coupling gratings, there are several possibilities. As one possible embodiment, one or more measurement areas of an array of measurement areas are provided on a grating structure (c) or (c').

As another possible embodiment, several or all arrays of measurement areas are provided on a common grating structure (c) or (c'). Evanescent field measurement platforms of this type, with one or more grating structures covering extended parts of the surface, with arrays of measurement areas provided thereon, have been described in more detail, with further embodiments that are also suitable for an analytical chip according to the present invention, in the international patent application WO 00/75644. These embodiments are especially advantageous when a very high surface density of measurement areas is desired, as the propagation (and hence possible optical cross-talk within the waveguiding layer (a)) of guided light in a direction perpendicular to the grating lines is limited to rather short distances, controllable mainly by the grating depth. As a special embodiment of this type, a superposition of two or more gratings with equal or different grating periods may be

provided, wherein the grating lines preferably are oriented other than in parallel, for example perpendicular to each other in case of two superimposed gratings.

Characteristic for a third type of embodiments of an analytical chip according to the invention is, that arrays of measurement areas are provided adjacent to or between grating structures (c) or (c').

There are many methods for the deposition of biological or biochemical or synthetic recognition elements, such as polynucleotides, on the optically transparent layer (a). For example, the deposition can be performed by physical adsorption or electrostatic interaction. In general, the orientation of the recognition elements is then of statistic nature. Additionally, there is the risk of washing away a part of the immobilized recognition elements, if the sample containing the analyte and reagents applied in the analysis process have a different composition. Therefore, it can be advantageous, if an adhesion-promoting layer (f) is deposited on the optically transparent layer (a), for immobilization of biological or biochemical or synthetic recognition elements. This adhesion-promoting layer should be transparent as well. In especial, the thickness of the adhesion-promoting layer should not exceed the penetration depth of the evanescent field out of the waveguiding layer (a) into the medium located above. Therefore, the adhesion-promoting layer (a) should have a thickness of less than 200 nm, preferably of less than 20 nm.

The adhesion-promoting layer can comprise, for example, chemical compoundsas known in the art, e.g. of the group comprising silanes, epoxides, functionalized, charged or polar polymers and "self-organized passive or functionalized mono- or multilayers", alkyl phosphates or alkyl phosphonates, and multifunctional block copolymers, such as poly(L)lysine / polyethylene glycols, and the like.

Laterally separated measurement areas can be generated by laterally selective deposition of biological or biochemical or synthetic recognition elements, such as polynucleotides, on the evanescent field measurement platform. Thereby, one or more deposition methods of the group of methods comprising "ink jet spotting", mechanical spotting by means of pin, pen or capillary, "micro contact printing", fluidically contacting the measurement areas with the biological or biochemical or synthetic recognition elements upon their supply in parallel or

crossed micro channels, upon exposure to pressure differences or to electric or electromagnetic potentials, can be applied.

In many cases the sensitivity of an analytical method is limited by signals caused by so-called nonspecific binding, i.e. by signals caused by the binding of the analyte or of other components applied for analyte determination or of compounds of the sample matrix, which are not only bound in the area of the provided immobilized biological or biochemical or synthetic recognition elements (e.g. polynucleotides), but also in areas of an evanescent field measurement platform that are not occupied by these recognition elements, for example upon hydrophobic adsorption or electrostatic interactions. Therefore, it is advantageous, if compounds, that are "chemically neutral" towards the analyte and / or towards the sample matrix, are deposited between the laterally separated measurement areas, in order to minimize nonspecific binding or adsorption. As "chemically neutral" compounds such components are called, which themselves do not have binding sites for the recognition and binding of the analyte or of an analogue of the analyte or of a further binding partner in a multi-step assay (and also for compounds of the sample matrix) and which prevent, due to their presence, the access of the analyte or of its analogue or of the further binding partners (or of compounds of the sample matrix) to the surface of the evanescent field measurement platform. Such a "chemically neutral" compound should also minimize nonspecific adhesion to the surface areas where it is deposited.

It is of large advantage, if the adhesion-promoting layer is "chemically neutral" towards compounds other than the recognition elements for the analytes contained in the sample, i.e., reduces nonspecific interaction with these compounds. For example, multifunctional block copolymers, such as poly(L)lysine / polyethylene glycols of adequate grafting ratio, are characterized by this favorable property.

For more general cases, where a minimization of nonspecific binding effects cannot be provided by an adhesion-promoting layer, it is preferred, that compounds which are "chemically neutral" towards the analytes and / or towards other compounds contained in the sample matrix, preferably of the groups comprising, for example, albumines, especially bovine serum albumine or human serum albumine, fragmentated natural or synthetic DNA, such as from herring or salmon sperm, not hybridizing with polynuleotides to be analyzed, or

uncharged but hydrophilic polymers, such as polyethyleneglycols or dextranes, are deposited between the laterally separated measurement areas.

On an analytical chip according to the invention, a very large total number of discrete measurement areas can be provided. It is possible to arrange more than 10,000, even up to 1,000,000 measurement areas in 2-dimensional arrangement. A single, individual measurement area can typically have an area between 0.001 mm^2 – 6 mm^2, whereby different measurement areas can have different size.

The measurement areas can be provided at a density of more than 10, preferably of more than 100, most preferably of more than 1000 measurement areas per square centimeter.

Characteristic for another preferred embodiment of an analytical chip according to the invention is, that the surface with the discrete measurement areas with immobilized polynucleotides forms the inner bottom surface of one or more sample compartments for receiving one or more samples to be analyzed for 16S-rRNA.

Thereby, it is preferred, that the one or more sample compartments are designed to accommodate a sample volume of less than 50 μl each, and that the inner bottom surface of a sample compartment is larger than 10 mm^2. Different embodiments of sample compartments, that are adequate to be formed with an analytical chip according to the invention, are described in the international patent applications WO 01/13096 and WO 01/43875, which are therefore incorporated in this patent application in their full entirety. Embodiments with a reservoir connected to the outlet of a flow cell, to receive exiting liquid, as described in WO 01/43854, appear especially useful, when sequentially several reagents or washing solutions have to be flown over the surface carrying the immobilized specific recognition elements (e.g. polynucleotides) and eventually 16S-rRNA bound respectively hybridized with them.

Grating structures (c) and optional addition grating structures (c') may be located within a sample compartment.

If the parts of the side walls touching the surface of the analytical chip, or a connecting material, such as a glue, between these side walls and the analytical chip, are optically

transparent (in the sense as defined above), then the grating structures (c) and optional additional grating structures (c') may also be located outside the sample compartments.

It is advantageous if the grating lines are oriented essentially in parallel to a pair of the side walls of the sample compartments, in order to reduce disturbing effects of reflections or scattering, especially when the grating structures are located outside the sample compartments. The grating structures (c) or (c') can be limited in their lateral extension on the analytical chip surface to the length of the parallel side walls of the sample compartments. However, they can also extend over the range of multiple or all sample compartments, for example along the whole width of an analytical chip (as defined) in the Example I.A.1 of this patent application.

For consecutive measurement of different arrays of measurement areas, it is of large advantage if the conditions for coupling of excitation light into an evanescent field measurement platform provided with an in-coupling grating vary only very little over extended distances, for example, if the coupling angle varies less than by 0.1°/cm in parallel to the grating lines. Evanescent field measurement platforms of such a high precision have been described in the international patent applications WO 01/55691 and WO 01/55760, which are incorporated in this patent application.

Another subject of the invention is an analytical method for the simultaneous determination of one or more different bacterial 16S-rRNA in a liquid sample, comprising providing an analytical chip comprising

- an evanescent field measurement platform, e.g. an optical waveguide, as a solid carrier and
- a plurality of specific recognition elements immobilized in discrete measurement areas of known location forming an array of measurement areas on said evanescent field measurement platform,

wherein

- a multitude (i.e. 2 or more) of different specific recognition elements is immobilized in discrete measurement areas for the recognition and detection of each different 16S-rRNA, different recognition elements being specific for

different subsequences of the 16S-rRNA to be detected, which are not directly adjacent and not overlapping in the sequence of said 16S-rRNA,

- a liquid sample, not being subjected to an amplification (e.g. by polymerase chain reaction PCR or linear amplification "T7") of the polynucleotide sequences contained therein, is brought into contact with the array under conditions allowing for binding (respectively hybridization) of 16S-rRNA contained in the sample with the corresponding specific recognition elements immobilized in the measurement areas
- changes of electro-optical signal caused by a successful binding (respectively hybridization) on the measurement areas of the evanescent field measurement platform are measured with one or more detectors, and
- the presence of a bacterium to be detected is determined from the whole of signals from those measurement areas occupied by immobilized specific recognition elements dedicated for the specific detection of said bacterium.

It is characteristic for the analytical method according to the invention, that it requires a lower number of required work-up steps, which are each associated with the risk of the introduction of experimental error and variation, than the known methods. As a consequence, the reliability and confidence into the results of an analytical method according to the invention, is considerably increased in comparison to the known methods. As a further consequence, the analytical chip according to the invention allows for a simultaneous quantitative determination of one or more different bacterial 16S-rRNA in a liquid sample, i.e. with an experimental variation of less than 50 %, preferably of less than 20 %, most preferably of less than 10 %. Thereby, the achievable low degree of experimental variation is of course dependent on the amount of available 16S-rRNA to be detected (i.e. a lower variation can be achieved if more of the 16S-rRNA to be detected is available). In contrast to the known analytical equipment and analysis methods based thereon, especially due to the lower number of required work-up steps (especially of biological / and or biochemical work-up steps typically inducing a large variability of measurement results) even a quantitative determination of the amount respectively concentration of the one or more different bacteria in the original sample from where the liquid sample containing said one or more different 16S-rRNA have been derived, is enabled.

It is preferred that the one or more bacterial 16S-rRNA to be detected are derived from bacteria selected from the group comprising e.g.: *Achromobacter xylosoxidans, Acinetobacter baumannii, Acinetobacter calcoaceticus, Acinetobacter junii, Acinetobacter wolfii, Actinobacillus sp, Actinomyces israelii, Actinomyces meyeri, Actinomyces odontolyticus, Actinomyces sp, Aerococcus viridans, Aeromonas caviae, Aeromonas hydrophilia, Aeromonas sobria, Agrobacterium radiobacter, Alcaligenes denitrificans, Alcaligenes faecalis, Alcaligenes sp, Alcaligenes xylosoxydans, Bacillus sp, Bacteroides bivius, Bacteroides buccae, Bacteroides caccae, Bacteroides denticola, Bacteroides disiens, Bacteroides distasonis, Bacteroides fragilis, Bacteroides oralis, Bacteroides oris, Bacteroides ovatus, Bacteroides stercoris, Bacteroides thetaiotomicron, Bacteroides uniformis, Bacteroides ureolyticus, Bacteroides vulgatus, Bifidobacterium sp, Bordetella bronchiseptica, Brucella melitensis, Burkholderia cepacia, Burkholderia picketti, Burkholderia pseudomallei, Campylobacter coli, Campylobacter fetus, Campylobacter jejuni, Campylobacter sp, Capnocytophaga canimorsus, Capnocytophaga ochracea, Capnocytophaga sp, Chryseomonas luteola, Citrobacter amalonaticus, Citrobacter braakii, Citrobacter diversus, Citrobacter freundii, Citrobacter koseri, Citrobacter sp, Clostridium bifermentans, Clostridium butyricum, Clostridium clostridiiforme, Clostridium paraputrificum, Clostridium perfringens, Clostridium ramosum, Clostridium septicum, Clostridium tertium, Clostridiumi innocuum, Comamonas acidovora, Corynebacterium aquaticum, Corynebacterium bovis, Corynebacterium jeikeium, Corynebacterium minutissimum, Corynebacterium sp, Eikenella corrodens, Empedobacter brevis, Entereococcus casseliflavus, Enterobacter aerogenes, Enterobacter agglomerans, Enterobacter amnigenus, Enterobacter cloacae, Enterococcus avium, Enterococcus durans, Enterococcus faecalis, Enterococcus faecium, Enterococcus gallinarium, Enterococcus raffinosus, Escherichia coli, Eubacterium aerofaciens, Eubacterium lentum, Eubacterium limosum, Flavobacterium breve, Flavobacterium meningosepticum, Flavobacterium sp, Fusobacterium sp, Fusobacterium mortiferum, Fusobacterium necrophorum, Fusobacterium nucleatum, Fusobacterium varium, Gardnerella vaginalis, Gemella haemolysans, Gemella morbillorum, Gemella sp, Haemophilus aphrophilus, Haemophilus influenzae, Haemophilus parainfluenzae, Haemophilus paraphrophilus, Hafnia alvei, Kingella sp, Klebsiella ornithinolytica, Klebsiella oxytoca, Klebsiella ozaenae, Klebsiella pneumoniae, Kluyvera sp, Lactobacillus acidophilus, Lactobacillus catenaforme, Lactococcus cremoris, Lactococcus lactis, Legionella pneumophila, Leptotrichia buccalis, Leuconostoc sp, Listeria monocytogenes, Moraxella catarrhalis, Moraxella osloensis, Moraxella phenylpyruvica, Moraxella sp, Morganella*

morganii, Mycobacterium avium, Mycobacterium genavense, Mycobacterium tuberculosis, Mycobacterium avium-intracellulare, Mycoplasma sp, Myroides odoratum, Neisseria cinerea, Neisseria flavescens, Neisseria meningitidis, Neisseria mucosa, Neisseria sp, Neisseria subflava, Nocardia asteroides, Nocardia sp, Ochrobactrum anthropi, Pasteurella multocida, Peptostreptococcus anaerobius, Peptostreptococcus asaccharolyticus, Peptostreptococcus magnus, Peptostreptococcus micros, Peptostreptococcus prevotii, Prevotella bivia, Prevotella buccae, Prevotella loescheii, Propionibacterium acnes, Propionibacterium granulosum, Proteus mirabilis, Proteus penneri, Proteus vulgaris, Providencia rettgeri, Providencia sp, Providencia stuartii, Pseudomonas aeroginosa, Pseudomonas alcaligenes, Pseudomonas diminuta, Pseudomonas fluorescens, Pseudomonas paucimobilis, Pseudomonas putida, Pseudomonas sp, Pseudomonas stutzeri, Pseudomonas vesicularis, Salmonella enteritidis, Salmonella paratyphi, Salmonella typhi, Salmonella typhimurium, Serratia fonticola, Serratia marcescens, Serratia odorifera, Serratia sp, Shigella dysenteria, Shigella flexneri, Shigella sonnei, Sphingomonas paucimobilis, Staphylococcus aureus, Staphylococcus auricularis, Staphylococcus capitis, Staphylococcus caprae, Staphylococcus chromogenes, Staphylococcus cohnii, Staphylococcus epidermidis, Staphylococcus haemolyticus, Staphylococcus hominis, Staphylococcus intermedius, Staphylococcus kloosii, Staphylococcus lugdunensis, Staphylococcus saccharolyticus, Staphylococcus saprophyticus, Staphylococcus sciuri, Staphylococcus simulans, Staphylococcus warneri, Staphylococcus xylosus, Stenotrophomonas maltophilia, Stomatococcus mucilaginosus, Streptococcus acidiminimus, Streptococcus adjacens, Streptococcus agalactiae, Streptococcus anginosus, Streptococcus bovis, Streptococcus canis, Streptococcus constellatus, Streptococcus cremoris, Streptococcus crista, Streptococcus defectivus, Streptococcus dysgalactiae, Streptococcus equinus, Streptococcus equisimilis, Streptococcus intermedius, Streptococcus lactis, Streptococcus mitis, Streptococcus mutans, Streptococcus oralis, Streptococcus pneumoniae, Streptococcus pyogenes, Streptococcus salivarius, Streptococcus sanguis, Streptococcus alpha-hemolyticus, Streptococcus beta-hemolyticus, Veillonella parvula, Veillonella sp, Yersinia enterocolitica, and the like.

For one group of embodiments of an analytical method according to the invention, it is characteristic that the immobilized specific recognition elements are selected from the group comprising, e.g., natural and synthetically fabricated polynucleotides, polynucleotides with artificial bases and / or artifical carbohydrates, peptides, peptide nucleic acids ("PNA"s),

PNA's with artificial bases, locked nucleic acids ® (LNAs, DK-2950 Vedbaek, Denmark), proteins (e.g. antibodies), ribozymes, and aptamers.

According to the recognition elements, another group of embodiments of the analytical method can be distinguished, wherein the immobilized specific recognition elements are selected, from the group of antibiotics-based recognition elements comprising, e.g., macrolide antibiotics (e.g. erythromycin, azithromycin, streptogramin), aminoglycoside antibiotics (e.g. neomycin, paromomycin, lividomycin, gentamycin), and peptide antibiotics (e.g. thiostreptone, micrococcin).

Preferred, however, is an embodiment of the analytical method for the simultaneous determination of one or more different bacterial 16S-rRNA in a liquid sample, comprising providing an analytical chip comprising

- an evanescent field measurement platform, e.g. an optical waveguide, as a solid carrier and
- a plurality of polynucleotides immobilized in discrete measurement areas of known location forming an array of measurement areas on said evanescent field measurement platform,

wherein

- a multitude (i.e. 2 or more) of different polynucleotides is immobilized in discrete measurement areas for the detection of each different 16S-rRNA, the sequences of the immobilized polynucleotides being essentially complementary to different subsequences of the 16S-rRNA to be detected, which are not directly adjacent and not overlapping in the sequence of said 16S-rRNA,
- a liquid sample, not being subjected to an amplification (e.g. by polymerase chain reaction PCR or linear amplification "T7") of the polynucleotide sequences contained therein, is brought into contact with the array under conditions allowing a hybridization of 16S-rRNA contained in the sample with essentially complementary polynucleotides immobilized in the measurement areas
- changes of electro-optical signal caused by a successful hybridization on the measurement areas of the evanescent field measurement platform are measured with one or more detectors, and

- the presence of a bacterium to be detected is determined from the whole of signals from those measurement areas occupied by immobilized polynucleotides dedicated for the specific detection of said bacterium.

Potential target sequences of 16S-rRNA to be derived from the above listed bacteria have been published to a large extent already in the literature. Otherwise they can be determined by known sequencing methods. For obtaining an unambiguous hybridization pattern (respectively "binding pattern" = pattern of the binding signals when using specific recognition elements other than polynucleotides) for a certain 16S-rRNA to be detected, an optimum choice of the target subsequences to be detected is of high importance. For example, if a certain genus shall be detected, then subsequences will be selected which are characteristic for that genus of bacteria and common to all its species or strains, whereas for detection of a certain species subsequences characteristic for only that species should be chosen.

Preferably, immobilized polynucleotides for the detection of the bacterial 16S-rRNA have a length of 5 – 500, preferably of 10 – 100 bases.

In typical embodiments of the analytical method according to the invention, the plurality of immobilized polynucleotides comprises 2 – 20 different polynucleotides which are essentially complementary to different subsequences of the same bacterial 16S-rRNA to be detected.

Due to the high sensitivity provided by an analytical chip according to the invention, not requiring an amplification, in general a lower number of different immobilized polynucleotides (or more generally: of specific recognition elements) is required for the unambiguous detection of a certain 16S-rRNA than for known analytical chips, which in general can only be applied in combination with the amplification of the biological material contained in a sample. It is preferred, that the plurality of immobilized polynucleotides (more generally: of immobilized specific recognition elements) comprises less than 10, preferably less than 5 different polynucleotides (more generally: specific recognition elements) which are essentially complementary to (respectively can bind to) different subsequences of the same bacterial 16S-rRNA to be detected.

Thereby, it is preferred that bacterial genus and / or species and / or strain are determined with a plurality of less than 10, preferably of less than 5 different immobilized polynucleotides (more generally: specific recognition elements), that hybridize specifically with (respectively bind specifically to) subsequences of the 16S-rRNA of said genus or species or strain.

It can be advantageous, for example, to reduce diffusion times of the target analyte towards the immobilized specific recognition elements (e.g. polynucleotides), if the bacterial 16S-rRNA to be detected is fragmented into strands of less than 500, preferably of less than 200 base pairs length.

It is preferred that the evanescent field measurement platform, as the basis for the analytical chip used in the analytical method according to the invention comprises an optical waveguide. Thereby, the optical waveguide can be continuous or partitioned into discrete waveguiding areas.

Especially preferred is, if the optical waveguide is an optical film waveguide with a first optically transparent layer (a) on a second optically transparent layer (b) with lower refractive index than layer (a).

For the further embodiments of an evanescent field measurement platform to be applied in an analytical method according to the invention, the same preferences of different embodiments are valid as described above for the evanescent field measurement platform as part of an analytical chip according to the invention.

The high sensitivity provided to the analytical method according to the invention by the favorite properties of the evanescent field can further be improved, if the resulting signals from the arrays of measurement areas, representing specific binding patterns, are analyzed by special mathematical and / or statistical methods dedicated for improvement of signal-to-noise ratios. Preferred such mathematical and / or statistical methods take advantage of additional input that is known about the system, but cannot directly deduced from the raw data (e.g., the expectation of the occurrence of mutations or of increased or decreased occurrence of an analyte leading to resulting differences between signal patterns to be observed). Examples of such methods, for the detection of mutations, are described in US-patents No. 6,136,541 and 6,142,681, which are incorporated hereby in this application in their full entirety. The use of

adequate methods described therein for an improvement of signal-to-noise ratios is part of the analytical method according to the invention.

For the detection of the binding of bacterial 16S-rRNA to the corresponding measurement areas, where their specific recognition elements (e.g. polynucleotides) are immobilized, the change of the so-called effective refractive index resulting from molecular adsorption to or desorption from the waveguide can be used for analyte detection. This change of the effective refractive index is determined, in case of grating coupler sensors (when the arrays of measurement areas are located on a coupling grating), from changes of the coupling angle for the in- and / or out-coupling of light into or out of the grating coupler sensor. In case of interferometric sensors, analyte binding can be determined from changes of the phase difference between measurement light guided in a sensing branch and a referencing branch of the interferometer.

The aforesaid refractive methods have the advantage, that they can be applied without using additional marker molecules, so-called molecular labels. The disadvantage of these label-free methods, however, is, that the achievable detection limits are limited to pico- up to nanomolar concentration ranges, dependent on the molecular weight of the analyte, due to lower selectivity of the measurement principle, which is not sufficient for many applications of modern trace analysis, which can be disadvantageous especially for diagnostic applications.

For achieving still lower detection limits, luminescence-based methods appear as more adequate, because of higher selectivity of signal generation. Therefore, it is preferred that the detection of the presence of bacterial 16S-rRNA is based on the change of one or more luminescences, preferably of one or more fluorescences. Thereby, the luminescence (fluorescence) used for analyte detection is generated by luminescence (fluorescence) labels, which are bound to or associated with the 16S-rRNA to be detected.

It is preferred that said labels are bound to polynucleotides (in especial 16S-rRNA) to be determined in a sample by a chemical (non-enzymatic) conjugation method. The labels may, for example, be added directly to the original sample to be analyzed and can thus be bound directly to the single-stranded nucleic acid (16S-rRNA), without the necessity of a transcription process. For example, the labeling can be performed upon end-labeling of the nucleic acid. Preferably, these labels have excitation and emission wavelengths between 250

nm and 1100 nm. The labels can be selected from luminescent, functionalized or intercalating dyes (a large variety of them being well-known in the literature), and luminescent, functionalized nanoparticles ("quantum dots", see: W. C. W. Chan and S. Nie, "Quantum dot bioconjugates for ultrasensitive nonisotopic detection", *Science* 281 (1998) 2016 – 2018).

It is preferred, that in-coupling of excitation light into the optically transparent, waveguiding layer (a) of an optical film-waveguide as evanescent field measurement platform used for the analytical method according to the invention, towards the measurement areas located thereon, is performed using one or more grating structures (c), that are formed in the optically transparent layer (a).

Characteristic for the analytical method according to the invention is, that a pattern of said changes of electro-optical signal caused by a successful hybridization of a multitude of immobilized polynucleotides, in different measurement areas, dedicated for the detection of one or more 16S-rRNA, ("sample hybrization pattern" of said 16S-rRNA) to be determined in a sample, is established and recorded. When using specific recognition elements other than polynucleotides (such as proteins), a "sample binding pattern" of said 16S-rRNA is established and recorded.

Preferably, also a "reference hybridization pattern" (respectively a "reference binding pattern" when using specific recognition elements other than polynucleotides) is established and recorded by bringing a liquid sample containing a known amount of one or more different known 16S-rRNA into contact with said analytical chip under conditions allowing for hybridization (respectively binding) between said known 16S-rRNA and the corresponding multitudes of complementary immobilized polynucleotides (more generally: specific recognition elements). Said "reference hybridization patterns" (respectively "reference binding pattern") are typically stored in a data library.

The sample and the reference hybridization pattern (respectively sample and reference binding pattern) can be established, for example by bringing the sample and the reference probe into contact with the same array of measurement areas (e.g. simultaneously or sequentially using labels with different emission wavelengths and optionally also different excitation wavelengths) and measuring and recording the resulting signal intensities. Especially when a high reproducibility of the array fabrication of the analytical chip with the

one or more arrays of measurement areas provided thereon is established, the hybridization (respectively binding) patterns of a sample and a reference can also be determined on different arrays, which are then preferably part of the same analytical chip. For this embodiment, preferably the same label is used both for the sample and for the reference.

Preferably, the obtained "sample hybridization patterns" and "reference hybridization patterns" (respectively "sample binding pattern" and "reference binding pattern") are stored in a data format that is compatible with the format of existing data libraries. Thus, also published data can be used as "reference hybridization patterns" (respectively "reference binding patterns").

As a typical embodiment of an analytical method according to the invention, 16S-rRNA contained in a sample are determined by comparison of a sample hybridization (respectively binding) pattern and one or more reference hybridization (respectively binding) patterns, upon determining the degree of agreement between said sample hybridization (respectively binding) pattern and said reference hybridization (respectively binding) patterns.

The comparison can be based, for example, on normalized signal intensities or on the difference or ratio of a sample hybridization (respectively binding) pattern and a reference hybridization (respectively binding) pattern. It is important to note that the assignment of the experimentally observed hybridization (respectively binding) patterns to 16S-rRNA of a certain genus or species or strain is based on the degree of agreement with a reference pattern and not on absolute signal patterns. As a consequence, knowledge of all the subsequences of the 16S-rRNA is not required.

Said degree of agreement between said sample hybridization (respectively binding) pattern and said reference hybridization (respectively binding) patterns can be determined by statistical methods or by other mathematical methods, like hierarchical cluster analysis (HCA), principal component analysis (PCA), and artificial neural networks (ANN). Thereby, the degree of agreement between said sample hybridization (respectively binding) pattern and said reference hybridization (respectively binding) patterns can be determined by mathematical clustering methods.

Preferably, the degree of agreement between said sample hybridization (respectively binding) pattern and said reference hybridization (respectively binding) patterns is determined artificial neural networks.

The invention is explained and illustrated by the following example, without restriction of generality.

Example

This example is related to an analytical chip according to the invention for human diagnostics, capable for the simultaneous detection of bacterial 16S-rRNA from up to 50 different, clinically most relevant pathogenic bacteria for humans. Using sets of multiple immobilized, different polynucleotides for recognition of and hybridization with different subsequences of a 16S-rRNA out of the multitude of different 16S-rRNA derived from all 50 different bacteria, specific hybridization patterns can be established for each of them. The example is also related to an analytical method according to the invention, using said analytical chip, for assignment of an observed and recorded hybridization pattern to a certain bacterium.

I.

A) Analytical Chip

1. Evanescent field measurement platform

An evanescent field measurement platform with the exterior dimensions of 57 mm width (in parallel to the grating lines of a grating structure (c) modulated in a layer (a) of the measurement platform) x 14 mm length (perpendicular to the lines of the grating structure) x 0.7 mm thickness is used. This evanescent field measurement platform can be combined with a plate of polycarbonate provided with recesses open towards said measurement platform and openings as fluid inlets towards its opposite side. Thereby the evanescent field measurement platform forms the inner bottom surface of an array of sample compartments, which are arranged (in this example) as a linear row of sample compartments with the interior dimensions of 5 mm width x 7 mm length x 0.15 mm height (above the evanescent field measurement platform). Combination of the polycarbonate plate with the evanescent field measurement platform as the base plate can, for example, be performed by gluing in such a way, that the recesses are tightly sealed against each other. Various embodiments of arrangements of sample compartments that can be generated using an analytical chip according to the invention are described in international patent applications WO 00/113096 and WO 00/143875, which are incorporated in this application in their full entirety. In this example, a linear arrangement of sample compartments is provided in such a form that can be inserted into a carrier ("meta carrier") with the footprint of standard microtiter plates (about 85 mm x 127 mm), the pitch of the inlets along one row of sample compartments being

compatible with the pitch of the wells of a standard microtiter plate. In this preferred embodiment, the outlet of each sample compartment is fluidically connected to a reservoir as part of this sample compartment (flow cell) arrangement, for receiving liquid exiting the sample compartment. Thus, for example, washing steps can be performed without the need of emptying the sample compartments in between.

The evanescent field sensor platform as part of analytical chip according to the invention is provided as optical thin-film waveguide with a first optically transparent layer (a) in a second optically transparent layer (b) with lower refractive index than layer (a). The material of layer (a) ("substrate material") consists of AF 45 glass (refractive index n = 1.52 at 633 nm). A pair of surface relief gratings ((c), (c')) is modulated in the substrate surface, on which during the further production process the second optically transparent and waveguiding layer (a) is deposited. Upon the deposition steps, the grating structures are reproduced in both the surface of layer (a) contacting layer (b) and into the opposite surface. The lines of the two grating structures (c), for coupling of excitation light into layer (a), and (c'), for coupling out light guided in layer (a), are oriented in parallel to the width of the evanescent field measurement platform, extending over the whole width. The grating period is 318 nm, the grating depth (12 +/- 3) nm. The distance between the two gratings is 9 mm, their length (in parallel to the length of the evanescent field measurement platform) 0.5 mm. The distance between the incoupling and the outcoupling grating of the pair of gratings is selected in such a way, that incoupling of excitation light can be performed within the base area of a sample compartment, formed by the combination of the evanescent field measurement platform with a polycarbonate plate as described above, whereas the outcoupling is performed outside of the sample compartments. The waveguiding, optically transparent layer (a) consists of Ta_2O_5 and has a thickness of 150 nm and a refractive index of 2.15 at 633 nm.

The sample compartments formed by the combination of the evanescent field measurement platform and the polycarbonate plate are provided with conical openings at the inner boundary surface opposite to the base plate, extending through the polycarbonate plate and thus allowing for filling or emptying the sample compartments upon inserting standard (micro) pipette tips.

As a preparation for the immobilization of the polynucleotides for specific recognition of and hybridization with bacterial 16S-rRNA, the evanescent field measurement platform is cleaned

using organic and inorganic reagents (e.g. propanol and sulphuric acid, with intermediate steps of washing with water) upon ultra-sonication. These and all further processing steps including the immobilization of the polynucleotides or oligonucleotides are performed under clean room conditions. The cleaned evanescent field measurement platforms are dried and then stored under pollution-free conditions until further processing.

2. Deposition of an adhesion-promoting layer

For improving the stability of the immobilized polynucleotides on the surface of the evanescent field measurement platform, an adhesion-promoting layer is deposited on the surface of said measurement platform: It is silanized with a functionalized silane in the liquid phase (2 % v/v 3-Glycidyloxypropyl-trimethoxy silane in xylol). After washing and drying, the evanescent field measurement platforms are again stored under pollution-free conditions until further processing.

3. Capture probe selection and array design

Sequences of 16S-rRNA strands of selected bacteria can be derived, for example, from GenBank via direct access or various database aggregators. On average, five different 19-mer subsequences per bacterial 16S-rRNA were selected, for which complementary oligonucleotides were obtained as capture probes to be immobilized on the evanescent field measurement platform. The most important criteria for the capture probe selection were:

- maximum diversity of the probe sequences within the full set of capture probes
- optimum GC content
- bias of positioning the probes towards the 3'ends of the 16S-rRNA strands
- comparable melting points of the probe-target (16S-rRNA) complexes to be formed (ideally similarity of melting points within +/- 1.5°)
- selection of "stable" regions of the target 16S-rRNA, with rare occurrence of mutations, as subsequences to be probed, in order to avoid high molecular variability

The design of an array capable for the determination of 21 different microorganisms using 272 different 19-mer capture probes (used for the correlation between hybridization patterns and identification of bacteria, example I.B.4), with 16 columns of measurement areas arranged in 18 rows, is depicted in Figure 1. The related sequences are listed in Table 1.

4. Immobilization of polynucleotides as specific recognition elements

In this example, without restriction of generality, single-stranded 19-mer oligonucleotides, which have been functionalized with amino groups at their 5'ends, are used as recognition elements for specific recognition of and hybridization with essentially complementary 16S-rRNA to be detected in a supplied sample. The oligonucleotides are provided at a concentration of 50 μM in carbonate buffer (200 mM) and deposited on the silanized surface of the evanescent field measurement platform in discrete measurement areas using a commercial spotter (Virtek, Eurogentecs, Seraing, Belgium). Up to twelve hours are allowed for covalent binding of the recognition elements and drying of the surface.

B) Analytical Method

1. Sample preparation

The sample preparation described here is used as a model system for samples to be taken and the material to be further processed from a whole blood sample in a real diagnostic application.

The bacteria to be determined are cultivated in a sugar-containing cultivation broth (in order to obtain enough material necessary for reference measurements using established methods requiring relatively large sample amounts). Then they are precipitated ("pelleted") from the culture medium by centrifugation. The bacterial cell walls are disrupted. The whole contained RNA ("total RNA") is subsequently isolated, using a commercial RNeasy Kit (Qiagen GmbH, Hilden, Germany).

The isolated "total RNA" is labeled with a rhodamine dye using a commercial "rhodamine nucleic acid labeling kit" (Kreatech Diagnostics, Amsterdam, The Netherlands). The kit

comprises a platinum complex with two active binding sites ("Universal Linkage System", ULS) for binding the fluorescence label (in this example the rhodamine dye) and the purin bases of the nucleic acids to be labeled.

This is an example of a "chemical" labeling method (by covalent binding), which is superior, in terms of reproducibility and labeling efficiency, to enzymatic labeling methods, for example by means of reverse transcriptase, as there is no dependence on enzymatic activity. Unbound fluorescence labels (respectively "ULS complexes) are removed upon processing with the RNeasy Kit, followed by a fragmentation step using T4 polynucleotide kinase that yields fragments typically in the range of 30 – 130 bases. The products were further purified using Centri-Sep columns (Princeton Separations, Adelphia, NJ, USA).

2. Hybridization with rhodamine-ULS-labeled total RNA

The rhodamine-ULS-labeled total RNA, containing the (labeled) 16S-rRNA to be detected besides other polynucleotides, such as mRNA, tRNA, etc., is diluted with water and hybridization buffer "2 x ZB1" (300 mM NaCl / 30 mM sodium citrate, pH 7.5) in such a way that a final volume of 25 μl is obtained, at a buffer concentration of "1 x ZB1" (150 mM NaCl, 15 mM sodium citrate, pH 7.5). The amounts of rhodamine-ULS-labeled total RNA, that are available for hybridization with the corresponding immobilized polynucleotides as capture probes of a total array of measurement areas, are between 2 ng and 500 ng. An amplification of the biological material (available total RNA) is not performed.

The prepared sample solution containing the labeled RNA is filled into a sample compartment housing an array of discrete measurement areas with 19-mer oligonucleotides immobilized on the silanized evanescent field measurement platform forming the bottom of the sample compartment. Hybridization of the immobilized capture probes with essentially complementary subsequences of 16S-rRNA contained in the sample is allowed for an incubation period of 60 minutes.

In order to increase the specificity of the hybridization, i.e., to minimize the amount of hybridization between sequences that are not completely complementary to each other, and especially to prevent unspecific hybridization, the hybridization step is performed under

"stringent conditions", for example at elevated temperature close to the melting temperature of the RNA to be detected (in this example at 50°C).

For further increase of selectivity, the analytical chip with the formed hybrids is then washed under "increasingly stringent conditions" (temperature: 20°C), first in washing buffer 1 (150 mM NaCl / 15 mM sodium citrate, pH 7.5, with 0.1 % SDS (sodium dodecyl sulfate)) for 5 minutes, then for 5 minutes in washing buffer 2 (15 mM NaCl / 1.5 mM sodium citrate, pH 7.5, with 0.1 % SDS), and finally for another 5 min in washing buffer 3 (15 mM NaCl / 1.5 mM sidium citrate, pH 7.5), taking thereby advantage of the reservoirs integrated on the analytical chip according to the invention (see example I.A.1).

"Increasingly stringent condition" shall mean that the dissociation of hybrids formed between not completely complementary polynucleotide sequences (with one or more mismatches between the formed pairs) is enhanced at decreasing concentration of positively charged ions in the buffer solution, as well as with decreasing concentration of detergents, which thus results in an increase of the selectivity of the method.

After termination of the hybridization and the subsequent washing steps, the ensemble of a row of sample compartments formed by the analytical platform, carrying the hybridized labeled 16S-rRNA bound from the sample, and by the polycarbonate plate combined with it, is inserted into a "meta carrier" (see Example A.1) and then inserted into a ZeptoREADER™ for excitation and detection of luminescence signals emanating from the measurement areas, (see below, Example I.B.3.) especially resulting from the binding of luminescently labeled 16S-rRNA on the corresponding measurement areas, and for the laterally resolved detection of the background signal intensities. During this measurement, the sample compartments are filled with buffer washing buffer 2.

3. Analytical system and measurement method

Fluorescence signals from different complete arrays of measurement areas, arranged at a 9 mm pitch (compatible to the pitch of standard microtiter plates) are measured sequentially using a ZeptoREADER™ (Zeptosens AG, Witterswil, Switzerland). For measurement of each array of measurement areas, the analytical chip according to the invention is adjusted for

fulfillment of the resonance condition for incoupling of light into the waveguiding tantalum pentoxide layer and for maximization of the excitation light available in the measurement areas.

Then a user-definable number of images of the fluorescence signals emanating from an array is generated for each array, wherein different exposure times can be chosen (typically in the range of 1 to 60 sec). The excitation wavelength for the measurements of the present example is 635 nm. Detection of the fluorescence light is performed using a cooled CCD camera at the emission wavelength of the fluorescence label upon using an interference filter (transmission (670 +/- 20) nm) positioned in front of the camera objective, for discrimination of scattered light at the excitation wavelength. The generated fluorescence images are automatically recorded on the hard disk of the control computer (for controlling the operation of the ZeptoREADER). Further details of the optical system (ZeptoREADER™) are described in the international patent application PCT/EP 01/10012, which is incorporated in this patent application in full entirety.

4. Data analysis and referencing

The medium signal intensity emanating from the measurement areas (spots) is determined using an image analysis software (ZeptoVIEW™, Zeptosens AG, CH-4108 Witterswil, Switzerland), which allows to analyze the fluorescence images of a multitude of arrays of measurement areas semi-automatically.

The raw data obtained from the individual pixels of the CCD (charge-coupled device) camera form a two-dimensional matrix of the digitized measurement data, with the measured intensity as the measurement value of a pixel corresponding to the surface section of the analytical chip imaged onto said pixel. For data analysis, at the beginning a two-dimensional (coordinate) net is superimposed over the image points (pixel values) in such a way that each measurement area (spot) is contained in an individual, two-dimensional net element. Within this net element, an "analysis element" (area of interest, "AOI")) is assigned to each spot, with a geometry optimized for matching the spot geometry. These AOIs can have any geometric form, for example circular form. The location of the AOIs in the two-dimensional net is individually optimized as a function of the signal intensity recorded by the

corresponding pixels. Dependent on the definitions set by the user, the initially defined radius of an AOI can be preserved or can be re-adjusted according to the geometry and size of a given spot. For example, the arithmetic average of the pixel values (signal intensities) can be determined as the mean gross signal intensity of every spot.

The background signals are determined from the signal intensities measured between the spots. For this purpose, for example, further circles can be defined, which are concentric with a given circular spot (and the assigned "spot AOI"), but have a larger radius. Of course, the radii of these concentric circles have to be smaller than the distance between adjacent spots. Then, for example, the region between the "spot AOI" and the first larger concentric circle can be disregarded, and the region between said first larger and a second still larger concentric circle can be defined as the AOI for the background determination ("background AOI"). It is also possible to define regions between adjacent spots, preferably located in the middle between adjacent spots, as AOIs for the determination of the background signal intensities. From these signal values the average background signal can then be determined in analogous way as described above, for example as the arithmetic average of the pixel values (signal intensities) of the chosen "background AOI". The average net signal intensity can then be determined as the difference between the local average gross and the local average background signal intensity.

5. Correlation of hybridization patterns and identification of bacteria

The data sets derived from the analyzed images of the hybridization patterns characteristic for different 16S-rRNA applied on the analytical chip are stored on a computer hard-disk in a spread sheet format. For correlation with the appropriate genus and / or species information, the entirety of the resulting data sets (that can already be regarded as a data library) is analyzed using hierachical cluster analysis. If required, the information stored there can be reduced using principal component analysis and further analyzed e.g. by using learning artificial neural networks

The hybridization patterns such as shown in Fig. 2 (from a smaller-sized array of 12 x 12 measurement areas) demonstrate the possibilities, dependent on the specifity of the target

subsequences of 16S-rRNA to be detected by immobilized complementary oligonucleotides, to determine a common genus and to differentiate, between different geni (*Staphylococcus*, Fig. 2 left and center, versus *Pseudomonas*, Fig. 2, right), as well as to differentiate between different species of the same genus (*Staphylococcus epidermidis*, Fig. 2 left, versus *Staphylococcus aureus*, Fig. 2, center). With the array shown in this example, parts of the array showed a similar hybridization pattern for the two different *staphylococcus* species, whereas in other parts of the array (especially concerning the upper left corner of Figure 2, left and center) considerable differences are observed.

Fig. 3 shows the full clustered pattern of data generated in 210 experiments, using the analytical chip described in Example I.A.3, for the determination of 21 different microorganisms using 272 different 19-mer capture probes. Clustering of the data was performed using the Average Linkage (UPGMA) variant of hierarchical cluster analysis. The y-axis shows the dendrogram of the clustered probes, the x-axis the different hybridization experiments ordered according to bacterium species and grouped for repetitive experiments.

The section enlargement of Fig. 4a shows – on the example of *Pseudomonas aeruginosa* ("Ps aerug") – the strong correlation of probes selected for *Peudomonas aeruginosa* and the high signal intensity obtained in all experiments (light gray colors representing high signal intensities, in contrast to dark colors representing low signal intensities), where *Pseudomonas aeroginosa* 16S-rRNA is present, in contrast to experiments, e.g. where *Enterococcus faecalis* ("Efaecal") or *Streptococcus agalactiae* ("Stagal") are determined.

The section enlargement of Fig. 4b highlights the probes indicative for the bacterial genus *Staphylococcus* – shown on the example of hybridization with *Staphylococcus aureus* ("St aureu") and *Staphylococcus epidermidis* ("St epide"), which leads to high signal intensities.

Running Number	Array Column	Array Row	Short Name	19mer Probe Sequence	Complete name
1	1	1	Ecloac_411	ACGTCAATTGCTGCGGTTA	Enterobacter cloacae
2	1	2	SA_15	AGCAAGCTTCTCGTCCGTT	Staphylococcus aureus
3	1	3	Buffer	Buffer	Buffer
4	1	4	Buffer	Buffer	Buffer
5	1	5	Buffer	Buffer	Buffer
6	1	6	Buffer	Buffer	Buffer
7	1	7	Buffer	Buffer	Buffer
8	1	8	Buffer	Buffer	Buffer
9	1	9	Buffer	Buffer	Buffer
10	1	10	Buffer	Buffer	Buffer
11	1	11	Buffer	Buffer	Buffer
12	1	12	Buffer	Buffer	Buffer
13	1	13	Buffer	Buffer	Buffer
14	1	14	Buffer	Buffer	Buffer
15	1	15	Buffer	Buffer	Buffer
16	1	16	Buffer	Buffer	Buffer
17	1	17	Buffer	Buffer	Buffer
18	1	18	Buffer	Buffer	Buffer
19	2	1	Ecoli_70	AGCAAGCCCTTCTGCTGTT	Escherichia coli
20	2	2	Ecoli_1093	GGCAGTCTCTCTTTGAGTT	Escherichia coli
21	2	3	Ecoli_1251	TCAGACTACGCACGACTTT	Escherichia coli
22	2	4	Efaec_137	GCCATGCGGCATAAACTGT	Enterococcus faecalis
23	2	5	Efaec_159	CGAAAGCGCCTTTCACTCT	Enterococcus faecalis
24	2	6	En_faeca_436	AGATACCGTCAGGGGACGT	Enterococcus faecalis
25	2	7	Kpn_378	TTCCTCCCCACTGAAAGTG	Klebsiella pneumoniae
26	2	8	Kpn_415	GGTAACGTCAATCGCCAAG	Klebsiella pneumoniae
27	2	9	Kpn_419	TGCGGGTAACGTCAATCGC	Klebsiella pneumoniae
28	2	10	St_epide_137	TCACTATTGAACCATGCGG	Not_Staph aureus
29	2	11	St_aureu_420	CCGTCAAGATGTGCACAGT	Not_Staph epidermidis
30	2	12	Psae_129	GATCCCCCACTTT	Pseudo aeruginosa

				CTCCCT	
31	2	13	StPn_126	TGTCATGCAACA TCCACTC	Streptococcus pneumoniae
32	2	14	StPn_159	CGTGAACGTAGT GATGGTC	Streptococcus pneumoniae
33	2	15	Pac_153	TTTCAAAGCCGC CAACCCC	Propionibacterium acnes
34	2	16	Psae_100	GCGGTATTAGCG CCCGTTT	Pseudo aeruginosa
35	2	17	Psae_121	ACTTTCTCCCTCA GGACGT	Pseudo aeruginosa
36	2	18	Pac_17	CCCACAAAAGCA GGGCCTT	Propionibacterium acnes
37	3	1	St_pneum_159	CTGGTAGTGATG CAAGTGC	Streptococcus pneumoniae
38	3	2	St_pneum_160	TCTGGTAGTGAT GCAAGTG	Streptococcus pneumoniae
39	3	3	St_pneum_162	CATCTGGTAGTG ATGCAAG	Streptococcus pneumoniae
40	3	4	St_pyoge_136	TAAATTACTAAC ATGCGTT	Streptococcus pyogenes
41	3	5	St_pyoge_134	AATTACTAACAT GCGTTAG	Streptococcus pyogenes
42	3	6	St_pyoge_193	AATTGCACCTTTT AAATGA	Streptococcus pyogenic consensus
43	3	7	St_pyoge_590	TAACTTCAGACTT AAAGAA	Streptococcus pyogenic consensus
44	3	8	St_saliv_134	AATAAATGACAT GTGTCAT	Streptococcus salivarius
45	3	9	St_saliv_135	AAATAAATGACA TGTGTCA	Streptococcus salivarius
46	3	10	St_saliv_910	CTATCTCTAGAA ATAGCAT	Streptococcus salivarius consensus
47	3	11	St_saliv_910	CTATCTCTAGAA ATAGCAT	Streptococcus salivarius consensus
48	3	12	St_sangu_124	ATGCAATAATCA ATTTTAT	Streptococcus sanguis
49	3	13	St_sangu_144	GCATCTTTCAATT AATTAT	Streptococcus sanguis
50	3	14	Ecloac_120 (G)	GGTCTTGCGACG TTATGCG	Enterobacter cloac
51	3	15	All_Staph_175	CACTTTTGAACC ATGCGGT	Staph consencus
52	3	16	All_Staph_941	CACCCCAATCAT TTGTCCC	Staph consensus
53	3	17	Bfrag_173	GAACGCATCCCC ATCCTTT	Bacteroides fragilis
54	3	18	Eaggl_1131	TTCAGTAGTACG GGAATGC	Entero agglomerans

55	4	1	St_dysga_146	TTGCACCTTTTAA ATGAAA	Streptococcus dysgalactiae
56	4	2	St_equin_143	CTTCTTTCAAGCA TCTAAC	Streptococcus equinus
57	4	3	St_inter_420	GTATGAACTTTCC ATTCTC	Streptococcus intermedius
58	4	4	St_miti_40	TCTACTTGCATGT ATTAGG	Streptococcus mitis
59	4	5	St_miti_1006	AAAACTCTATCT CTAGAGC	Streptococcus mitis
60	4	6	St_mitis_187	ACCTTTTAAGTA AATGTCA	Streptococcus mitis consensus
61	4	7	St_mitis_187	ACCTTTTAAGTA AATGTCA	Streptococcus mitis consensus
62	4	8	St_mitis_193	AATTGCACCTTTT AAGTAA	Streptococcus mitis consensus
63	4	9	St_mitis_193	AATTGCACCTTTT AAGTAA	Streptococcus mitis consensus
64	4	10	St_mutan_155	CTTGCATCTTTCA ATCAAT	Streptococcus mutans
65	4	11	St_mutan_138	ATTATCATGCAA TAATTAA	Streptococcus mutans
66	4	12	St_mutan_529	TTTAACTTCAGAC TTACTA	Streptococcus mutans consensus
67	4	13	St_mutan_127	ATCTTTCAATCAA TTAACA	Streptococcus mutans consensus
68	4	14	St_pneum_136	ACTAGCTAATAC AACGTAG	Streptococcus pneumonia
69	4	15	St_pneum_136	ACTAGCTAATAC AACGTAG	Streptococcus pneumonia
70	4	16	St_pneum_585	AAAGCCTACTAT GGTTAAG	Streptococcus pneumonia consensus
71	4	17	St_pneum_125	GTCATGCAACAT CCACTCT	Streptococcus pneumoniae
72	4	18	St_pneum_126	TGTCATGCAACA TCCACTC	Streptococcus pneumoniae
73	5	1	St_kloos_163	TCTATAAGTGAT AGCAAGG	Staphylococcus kloosii
74	5	2	St_lugdu_372	AGAGTTTTACGA TCCTAAG	Staphylococcus lugdunensis
75	5	3	St_sapro_407	CACAGTTACTTA CACATTT	Staphylococcus saprophyticu
76	5	4	St_sapro_404	AGTTACTTACAC ATTTGTT	Staphylococcus saprophyticu
77	5	5	St_sciur_408	TTCAGTTACTAAC AAATTT	Staphylococcus sciuri
78	5	6	St_simul_408	GCATAGTTACTT ACATCCT	Staphylococcus simulans
79	5	7	St_warne_118	TTCAATATGTTAT	Staphylococcus

				CCGGTA	warneri
80	5	8	St_xylos_117	TCTAAATGTTATC CGGTAT	Staphylococcus xylosus
81	5	9	St_mucil_69	CAGAGTTAAAGG TAGGTTA	Stomatococcus mucilaginosus
82	5	10	St_agala_128	TAACATGTGTTA ATTACTC	Streptococcus agalactiae
83	5	11	St_agala_25	ATCAGTCTAGTG TAAACAC	Streptococcus agalactiae
84	5	12	St_agala_28	CTCATCAGTCTA GTGTAAA	Streptococcus agalactiae
85	5	13	St_angin_365	ACAGTATGAACT TTCCATT	Streptococcus anginosus consensus
86	5	14	St_angin_494	TTTCACTTCAGAC TTATCT	Streptococcus anginosus consensus
87	5	15	St_bovis_159	TGTGTTAAATGCT GTTATG	Streptococcus bovis consensus
88	5	16	St_bovis_575	CTTCAGACTTATT AAACCG	Streptococcus bovis consensus
89	5	17	St_bovis_995	CTTCCTATCTCTA GGAATA	Streptococcus bovis consensus
90	5	18	St_canis_133	GTTCTTAACATGT GTTAAG	Streptococcus canis
91	6	1	Ci_freun_949	TATCGAATTAAA CCACATG	Citrobacter freundi
92	6	2	Bu_cepac_389	TAGAACCAAGGA TTTCTTT	Burkholderia cepacia
93	6	3	Ba_dista_377	CTTATAAAAGAG GTTTACG	Bacteroides distasonis
94	6	4	Ac_meyer_111	CAGTGAATATCC AGTATTA	Actinomyces meyeri
95	6	5	St_epide_152	CAGCAAAACCGT CTTTCAC	Staphylococcus epidermidis
96	6	6	Se_marce_465	TCAATTGATGAG CGTATTA	Serratia marcescen
97	6	7	Ps_aerug_100	GCGGTATTAGCG CCCGTTT	Pseudomonas aeruginosa
98	6	8	Pr_mirab_416	GTAACGTCAATT GATAAGG	Proteus mirabilis
99	6	9	Pe_conse_617	TAGCAGTTTTAA ATGCTTA	PeptoStreptococcus consensus
100	6	10	Le_pneum_405	TTAATCAGCTCTT AACCTA	Legionella pneumophila
101	6	11	Kl_oxyto_401	ATAAGGTTATTA ACCTCAC	Klebsiella oxytoca
102	6	12	Ge_haemo_144	AACTTTTAAACA TCAACCA	Gemella haemolysans
103	6	13	St_epide_210	ACATCAGCGTCA GATACAG	Staphylococcus epidermidis

104	6	14	St_epide_214	TCGCACATCAGC GTCAGAT	Staphylococcus epidermidis
105	6	15	St_epide_134	CTATTGAACCAT GCGGTTC	Staphylococcus epidermidis
106	6	16	St_haemo_405	TAGTTACTTACAC GTATGT	Staphylococcus haemolyticus
107	6	17	St_homin_163	TCTATAAGTGAT AGCAGAG	Staphylococcus hominis
108	6	18	St_homin_163	TCTATAAGTGAT AGCAGAG	Staphylococcus hominis
109	7	1	En_aggl._1131	CGTAAGGGCATG ATGACTT	Enterobacter aggl.
110	7	2	Cl_ramos_137	CTTTGAGGCACTT TTAATA	Clostridium ramosum
111	7	3	Ci_freun_949	TATCGAATTAAA CCACATG	Citrobacter freundi
112	7	4	Ba_vulga_430	TCCTTATTCATAA AGTACA	Bacteroides vulgatus
113	7	5	Ba_conse_101 2	TTTCCACATAATT CAGTTG	Bacteroides consensus
114	7	6	Ac_meyer_112	CCAGTGAATATC CAGTATT	Actinomyces meyeri
115	7	7	St_cohni_115	TAAATGTTATCC GGCATTA	Staphylococcus cohnii
116	7	8	St_epide_64	CATGCGGTTCAA TATATTA	Staphylococcus epidermidis
117	7	9	Ps_aerug_11	TGTTCCTTCCTAT ATCTAC	Pseudomonas aeruginos
118	7	10	Pr_conse_1476	TTACCTTGTTACG ACTTAG	Propionibacterium consensus
119	7	11	Pe_conse_111 4	TGCTGGTAACTA AAGATAG	PeptoStreptococcus consensus
120	7	12	Le_pneum_40 3	AATCAGCTCTTA ACCTATC	Legionella pneumophila
121	7	13	Kl_pneum_41 5	GGTAACGTCAAT GAATAAG	Klebsiella pneumoniae
122	7	14	Ge_haemo_41 0	TGTATAGTTACTA CACAAT	Gemella haemolysans
123	7	15	Eu_limos_130	AAAACCATAATA TAAGGCT	Eubacterium limosum
124	7	16	En_faeca_42	GCCACTCCTCTTT CCAATT	Enterococcus faecalis
125	7	17	En_aggl._533	GGGGATTTCACA TCGACTT	Enterobacter aggl.
126	7	18	Cl_terti_141	GCTCCTTTAATTA CTTCTT	Clostridium tertium
127	8	1	Es_coli_475	CGCTGAAAGTAC GTGGCTT	Escherichia coli

128	8	2	En_faeca_1261	CTTTAAGAGATTTGCATGA	Enterococcus faecali
129	8	3	En_aggl._1071	CCTTTGAGTTCCCACCATT	Enterobacter aggl.
130	8	4	Cl_perfr_111	TCAACATTATGCGGTATTA	Clostridium perfringens
131	8	5	Ci_freun_1163	TTCCTCTAGTTTATCACTG	Citrobacter freundi
132	8	6	Ba_ureol_378	ATTCTTTCCTGATAAAAGG	Bacteroides ureolyticus
133	8	7	Ba_conse_1008	CACATAATTCAGTTGCAAT	Bacteroides consensus
134	8	8	Ac_junii_395	AAGAGTATTAGTCTCAGTA	Acinetobacter junii
135	8	9	St_aureu_16	AAGCAAGCTTCTCGTCCGT	Staphylococcus aureus
136	8	10	St_epide_62	TGCGGTTCAATATATTATC	Staphylococcus epidermidis
137	8	11	Ps_aerug_4	TCCTATATCTACGCATTTC	Pseudomonas aeruginos
138	8	12	Pr_conse_1469	GTTACGACTTAGTCCTAAT	Propionibacterium consensus
139	8	13	Pe_anaer_145	ACTTTGATATATCTACGAT	PeptoStreptococcus anaerobi
140	8	14	La_conse_85	AAGCACCATTCATTATTAA	Lactobacillus consensus
141	8	15	Ha_parap_125	TTTCATCTCTCGATTCTAC	Haemophilus paraphrophilus
142	8	16	Fu_variu_130	ATAGCTTTCATAACCAAAT	Fusobacterium varium
143	8	17	Es_coli_899	AACGCACATCATGCGTCTT	Escherichia coli
144	8	18	En_faeca_159	CGAAAGCGCCTTTCACTCT	Enterococcus faecalis
145	9	1	Ha_parai_400	AGTCTATTAAACTAAATGC	Haemophilus parainfluenzae
146	9	2	Fu_nucle_140	TAGCTTTCATAATTCTAAG	Fusobacterium nucleatum
147	9	3	Es_coli_1283	AACCGACTCCATGAAGTCG	Escherichia coli
148	9	4	En_faeca_1267	GAGAAGCTTTAAGAGATTT	Enterococcus faecali
149	9	5	En_aerog_400	CAAGGTTATTAACCTTAAC	Enterobacter aerogenes
150	9	6	Cl_perfr_108	ACATTATGCGGTATTAATC	Clostridium perfringens
151	9	7	Ci_freun_1164	CTTCCTCTAGTTTATCACT	Citrobacter freundi
152	9	8	Ba_theta_155	CCGAAATTCTTTA	Bacteroides

				ATAATA	thetaiotaomicro
153	9	9	Ba_conse_639	CAGTATCAACTG CAATTTT	Bacteroides consensus
154	9	10	Ac_conse_167	GAGATGATATCC GGTATTA	Acinetobacter consensus
155	9	11	Es_coli_70	AGCAAGCTTCTC GTCCGTT	Escherichia coli
156	9	12	St_epide_63	ATGCGGTTCAAT ATATTAT	Staphylococcus epidermidis
157	9	13	Ps_aerug_10	GTTCCTTCCTATA TCTACG	Pseudomonas aeruginos
158	9	14	Pr_acnes_418	AGTTAGCCGGTG CTTCTTT	Propionibacterium acnes
159	9	15	Pe_anaer_108	CATGTATTAGTA AACTTTT	PeptoStreptococcus anaerobi
160	9	16	La_conse_7	GGATCAAACTCT CATTTTA	Lactobacillus consensus
161	9	17	Ha_parap_417	ATTAACGTCAAT TTGTTGT	Haemophilus paraphrophilus
162	9	18	Fu_nucle_131	TAATTCTAAGAT GCCTTAA	Fusobacterium nucleatum
163	10	1	Pa_multo_404	GCTATCTATTTAA CAACAT	Pasteurella multocida
164	10	2	Kl_pneum_444	CGGGTAACGTCA ATCGATG	Klebsiella pneumoniae
165	10	3	Ha_parai_402	CTAGTCTATTAA ACTAAAT	Haemophilus parainfluenzae
166	10	4	Fu_necro_103	AAAACCATAATA TCCGGTA	Fusobacterium necrophorum
167	10	5	Es_col_865	AATTCGATTTGA GTTTTAA	Escherichiacol
168	10	6	En_conse_450	GATGAACATTCT ACTCTCA	Enterococcus consensus
169	10	7	En_aerog_399	AAGGTTATTAAC CTTAACG	Enterobacter aerogenes
170	10	8	Cl_parap_134	TTTAATTGCTACT TCATGC	Clostridium paraputrificum
171	10	9	Ci_amalo_408	AATGGCTAAGGT TATTAAC	Citrobacter amalonaticus
172	10	10	Ba_theta_155	CCGAAATTCTTTA ATAATA	Bacteroides thetaiotaomicro
173	10	11	Ba_cacca_156	ACCGAATTTCTTT AATATA	Bacteroides caccae
174	10	12	Ac_conse_167	GAGATGATATCC GGTATTA	Acinetobacter consensus
175	10	13	St_aureu_440	CGTGGCTTTCTGA TTAGGT	Staphylococcus aureus
176	10	14	Sa_enter_776	GTATATAATCCT GTTTGCT	Salmonella enteritidi

177	10	15	Ps_aerug_6	CTTCCTATATCTACGCATT	Pseudomonas aeruginos
178	10	16	Pr_acnes_17	CCCACAAAAGCAGGGCCTT	Propionibacterium acnes
179	10	17	Pa_multo_421	TAATTAACGTCAATGATGC	Pasteurella multocida
180	10	18	La_conse_1061	CTAAGGTTGTCAAAAGATG	Lactobacillus consensus
181	11	1	Pr_stuar_403	CGTTGATGGTATTAACATC	Providencia stuartii
182	11	2	Pr_acne_877	CAATTCCTTTGAGTTTTAG	Propionibacterium acne
183	11	3	Ne_flave_183	CCAACTAACTAATCAGATA	Neisseria flavescens
184	11	4	Kl_pneum_419	TGCGGGTAACGTCAATCGC	Klebsiella pneumoniae
185	11	5	Ha_influ_415	TTAACGTCAATTTGATGTA	Haemophilus influenzae
186	11	6	Fu_necro_133	CATCTAGCTTTCATGATTC	Fusobacterium necrophorum
187	11	7	Es_col_1404	TACTTCTTTTGGACGAATA	Escherichiacol
188	11	8	En_conse_1271	GAGAAGCTTTAAGAGATTA	Enterococcus consensus
189	11	9	Ei_corro_181	ACTAGCTAATCAGTTATCG	Eikenella corrodens
190	11	10	Cl_innoc_42	GCTCAGTCAATTTAAATTC	Clostridium innocuum
191	11	11	Ci_amalo_401	AAGGTTATTAACCTTAACC	Citrobacter amalonaticus
192	11	12	Ba_sterc_150	AATTCTTTAATAATCATCC	Bacteroides stercoris
193	11	13	Ba_cacca_119	TATGCTATCGGATATTAAT	Bacteroides caccae
194	11	14	Ac_conse_166	AGATGATATCCGGTATTAG	Acinetobacter consensus
195	11	15	St_aureu_185	TAGCTAATGCAGCGCGGAT	Staphylococcus aureus
196	11	16	Sa_conse_1014	ATTCTCATCTCTGAAAACT	Salmonella consensus
197	11	17	Pr_stuar_407	CAATCGTTGATGGTATTAA	Providencia stuartii
198	11	18	Pr_acnes_153	CCCCAACCGCCGAAACTTT	Propionibacterium acnes
199	12	1	St_aureu_150	GCAAGACCGTCTTTCACTT	Staphylococcus aureus
200	12	2	Sa_conse_1	TGATCAAACTCTTCAATTT	Salmonella consensus
201	12	3	Pr_vulga_404	GCTAAGAGTATT	Proteus vulgaris

				AATCTTA	
202	12	4	Ae_virid_407	AGGAGGACATAA GGTATTA	Aerococcus viridans
203	12	5	Ne_flave_184	ACCAACTAACTA ATCAGAT	Neisseria flavescens
204	12	6	Empty	Buffer	EMPTY
205	12	7	Ha_influ_414	TAACGTCAATTT GATGTAC	Haemophilus influenzae
206	12	8	Fu_morti_131	TATAGCTTTCATA TGAATT	Fusobacterium mortiferum
207	12	9	Es_col_672	GATCTCTACTGG AATTCTA	Escherichiacol
208	12	10	En_cloac_411	ACGTCAATTGCT GCGGTTA	Enterobacter cloacae
209	12	11	Ei_corro_183	CAACTAGCTAAT CAGTTAT	Eikenella corrodens
210	12	12	Cl_innoc_42	GCTCAGTCAATTT AAATTC	Clostridium innocuum
211	12	13	Ch_luteo_395	CAACGTATTAGG TTACAAC	Chryseomonas luteola
212	12	14	Ba_ovatu_136	ATATCATGCGAT ATTCGTA	Bacteroides ovatus
213	12	15	Al_faeca_95	CACTCTTTCGAGT AGTTAT	Alcaligenes faecalis
214	12	16	Ac_conse_166	AGATGATATCCG GTATTAG	Acinetobacter consensus
215	12	17	St_aureu_175	CACTTTTGAACC ATGCGGT	Staphylococcus aureus
216	12	18	Sa_conse_101 3	TTCTCATCTCTGA AAACTT	Salmonella consensus
217	13	1	Ae_hydro_410	ACAGTTGATACG TATTAGG	Aeromonas hydrophila
218	13	2	Ac_conse_125 2	TTTTGAGATTAGC ATCCTA	Acinetobacter consensus
219	13	3	St_aureu_1237	TCGCTGCCCTTTG TATTGT	Staphylococcus aureus
220	13	4	St_aureu_994	TATCTCTAGAGTT GTCAAA	Staphylococcus aureus
221	13	5	Pr_vulga_402	TAAGAGTATTAA TCTTAAC	Proteus vulgaris
222	13	6	Pe_prevo_16	AGAGATCATTTA AGCTTCA	PeptoStreptococcus prevotii
223	13	7	Mo_morga_40 1	AAGGTTATTAAC CTTGACA	Morganella morganii
224	13	8	Kl_pneum_42 9	GATGAGGTTATT AACCTCA	Klebsiella pneumonia
225	13	9	Ha_aphro_16	TACAAGTACTTA CCTGTTA	Haemophilus aphrophilus
226	13	10	Fu_morti_130	ATAGCTTTCATAT	Fusobacterium

				GAATTT	mortiferum
227	13	11	Es_col_436	ATGAGCAAAGTA TTAGACT	Escherichiacol
228	13	12	En_cloac_120	GGTCTTGCGACTT TATGCG	Enterobacter cloacae
229	13	13	Co_conse_176	TAAAGTATGGTG TCCTATC	Corynebacterium consensus
230	13	14	Cl_conse_200	ATGCGATACTCT GATATTA	Clostridium consensus
231	13	15	Ca_ochra_136	GCTTTAATAGTTG TGTGAT	Capnocytophaga ochracea
232	13	16	Ba_fragi_174	GGAACGCATCCC CATCCTT	Bacteroides fragilis
233	13	17	Ae_hydro_412	TCACAGTTGATA CGTATTA	Aeromonas hydrophila
234	13	18	Ac_conse_978	CTCTGGAAAGTT CTTACTA	Acinetobacter consensus
235	14	1	Ca_conse_718	TCAATTAATTGTT AGTAAT	Capnocytophaga consensus
236	14	2	Ba_fragi_1112	TGTTAGTAACTA AAGATAA	Bacteroides fragili
237	14	3	Ae_virid_439	GTGGCTTTCTGAT AAGATA	Aerococcus viridans
238	14	4	Ac_conse_428	TATTAACCAAAG TAGCCTC	Acinetobacter consensus
239	14	5	Sh_dysen_435	AAAGGTATTAAC TTTACTC	Shigella dysenteria
240	14	6	St_aureu_768	TATCTAATCCTGT TTGATC	Staphylococcus aureus
241	14	7	Pr_penne_413	ACGTCAATTGAT AAAGGTA	Proteus penneri
242	14	8	Pe_prevo_18	GAAGAGATCATT TAAGCTT	PeptoStreptococcus prevotii
243	14	9	Mo_morga_402	CAAGGTTATTAA CCTTGAC	Morganella morganii
244	14	10	Kl_pneum_516	TAATTCCGATTA ACGCTTA	Klebsiella pneumonia
245	14	11	Ha_aphro_17	GTACAAGTACTT ACCTGTT	Haemophilus aphrophilus
246	14	12	Fu_conse_877	ATTCCTTTGAGTT TCATAC	Fusobacterium consensus
247	14	13	Es_col_2	CATGATCAAACT CTCAATT	Escherichiacol
248	14	14	En_cloac_120	GGTCTTGCGACTT TATGCG	Enterobacter cloacae
249	14	15	Co_conse_956	CATCGAATTAAT CCACATG	Corynebacterium consensus
250	14	16	Cl_conse_632	TTTCACATCTGAC TTAAAT	Clostridium consensus

251	14	17	Ca_conse_228	TCTCCAACTAGCTAATAGA	Capnocytophaga consensus
252	14	18	Ba_fragi_162	CATCCTTTACCGGAATCCT	Bacteroides fragilis
253	15	1	CN_conse_243	TTACCAACTAGCTAATACG	CNS consensus
254	15	2	Cl_bifer_88	CCGTATTAGTATACCTTTC	Clostridium bifermentans
255	15	3	Bu_cepac_395	CTGTATTAGAACCAAGGAT	Burkholderia cepacia
256	15	4	Ba_fragi_448	CTGCACTTTATTCTTATAT	Bacteroides fragili
257	15	5	Ae_virid_70	GCTTATAGGTAGATTCCTT	Aerococcus viridans
258	15	6	Ac_calco_397	CTGAAGGTATTAACTTCAG	Acinetobacter calcoaceticus
259	15	7	Sh_dysen_981	GTATCTCTACAAGGTTCTG	Shigella dysenteria
260	15	8	St_aureu_446	CACAGTTACTTACACATAT	Staphylococcus aureus
261	15	9	Pr_penne_415	TAACGTCAATTGATAAAGG	Proteus penneri
262	15	10	Pe_micro_15	AGAATTTCCACAAAAATCA	PeptoStreptococcus micros
263	15	11	Mo_osloe_409	CAGGTAACGTCTAATCTAA	Moraxella osloensis
264	15	12	Kl_pneum_432	ATCGATGAGGTTATTAACC	Klebsiella pneumonia
265	15	13	Ge_morbi_148	AACCAACTTTTAAATATCT	Gemella morbillorum
266	15	14	Fu_conse_1261	CTAAGAATAGTTTTCTGAG	Fusobacterium consensus
267	15	15	En_faeci_1261	TTTAAGAGATTAGCTTAGC	Enterococcus faeciu
268	15	16	En_agglo_1282	GTAACATTCTGATTTACGA	Enterobacter agglomeran
269	15	17	Co_conse_177	CTAAAGTATGGTGTCCTAT	Corynebacterium consensus
270	15	18	Cl_clost_18	GAAAACTTCATCTTAATTG	Clostridium clostridiiforme
271	16	1	En_faeca_436	AGATACCGTCAGGGGACGT	Enterococcus faecalis
272	16	2	En_aggl._578	AGAACTCAAGCTGCCAGTT	Enterobacter aggl.
273	16	3	CN_conse_1455	TAGCTCCTAATAAATGGTT	CNS consensus
274	16	4	Ci_freun_1165	CCTTCCTCTAGTTTATCAC	Citrobacter freundi
275	16	5	Bu_cepac_393	GTATTAGAACCA	Burkholderia cepacia

276	16	6	Ba_fragi_453	AGGATTT ACATACTGCACT TTATTCT	Bacteroides fragili
277	16	7	St_aureu_15	AGCAGTTACTCT ACAATTT	Staphylococcus aureus
278	16	8	Ac_bauma_394	TAGGTATTAACT AAAGTAG	Acinetobacter baumannii
279	16	9	Se_marce_464	CAATTGATGAGC GTATTAA	Serratia marcescen
280	16	10	Ps_aerug_121	ACTTTCTCCCTCA GGACGT	Pseudomonas aeruginosa
281	16	11	Pr_mirab_417	GGTAACGTCAAT TGATAAG	Proteus mirabilis
282	16	12	Pe_micro_400	CGTCATTATCTTC TCATAG	PeptoStreptococcus micros
283	16	13	Mo_catar_26	ACTAAGTATCAG AAGCAAG	Moraxella catarrhalis
284	16	14	Kl_pneum_435	TCAATCGATGAG GTTATTA	Klebsiella pneumonia
285	16	15	Ge_morbi_406	TAGTTACTACAT ATCCATT	Gemella morbillorum
286	16	16	Fu_conse_1267	TCCGAACTAAGA ATAGTTT	Fusobacterium consensus
287	16	17	En_faeca_89	TTATCCCCCTCTG ATGGGT	Enterococcus faecalis
288	16	18	En_agglo_399	GATGAAGTATTA ATTTCAC	Enterobacter agglomeran

Table 1